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EACH number of ACTA PHARMACOLOGICA ET TOXICOLOGICA consists of about 5 printed sheets, 6 numbers forming a volume The numbers will be issued as soon as the articles submitted can be printed

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From the Institute of Occupational Health Oslo, Norway

The Effect of Chelating Agents on Biliary Excretion of Methyl Mercuric Salts in the Rat

By

Tor Norseth

(Received March 27, 1972, Accepted July 7, 1972)

bstract DL-penicillamine and dimercaptopropanol (BAL) increase the biliary xcretion of mercury after the injection of methyl mercuric chloride The effect sex dependent and dependent on the time interval between mercury injection id the treatment. The time dependent effect is related to the excretion of both iorganic mercury and the intact organo-mercurial The results indicate that a ombined use of chelating agent and the previously described resin which inter ipts the enterohepatic circulation should be valuable for the treatment of posure to organic or inorganic mercury compounds

ey words Methyl mercuric chloride – chelating agents – biliary excretion

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ENSSON & ULFVARSON (1967) found no antidote with life saving effect s in acute methyl mercuric hydroxide poisoning All the compounds caused changes in organ distribution or excretion pattern of mercury tribution of mercury after dimercaptopropanol treatment has been de- rated on exposure to both methyl mercuric salts and inorganic mer- BERLIN *et al* 1965, BERLIN & LEWANDER 1964) An increased brain of mercury was specifically noted Excretion was not increased after ire to methyl mercuric salts Mercury undergoes enterohepatic cir- in after an intravenous injection of methyl mercuric chloride into (NORSETH & CLARKSON 1971) The redistribution of mercury after ent with chelating agents may involve a biliary excretion mechanism nce cause changes in the enterohepatic circulation

w approach to the treatment of poisoning with methyl mercuric salts ggested recently, based on a possible interruption of the enterohepatic ion by a mercury absorbing resin which is not absorbed from the ntestinal tract (NORSETH & CLARKSON 1971) In this respect a sulph-

hydryl substituated polyvinyl resin was among others found useful (CLARKSON *et al* 1971)

If biliary excretion of mercury after exposure to methyl mercuric compounds could be increased by treatment with chelating agents, a combined treatment of chelating agent and resin should be tested. This paper reports the effect of several chelating agents on the biliary excretion of mercury after the intravenous injection of methyl mercuric chloride into the rat

Materials and Methods

Rats of both sexes (weighing about 200 g) of our own breed (Wistar) were used for the experiment The bile duct was cannulated under barbiturate narcosis (sodium pentobarbitone, mebumalum NFN), and the bile was collected in pre weighed vials The rats were kept asleep for 6 hours and the bile was collected in one hour samples A tracheal tube was inserted to keep free airways during the collection period and the body temperature was kept constant The rats were killed after 6 hours by blood collection from the abdominal aorta Blood and liver samples were collected for control purposes, but the results are not included in the paper

The biliary concentration of mercury was always recorded for 3 hours before the administration of the chelating agent This control period was necessary because the biliary excretion of mercury recorded either as concentration or as total excretion varied by more than a factor of two even between rats of the same breed and sex An effect of a chelating agent might thus easily be masked by variation between individuals A similar variation has been found previously for other metals when excretion during relatively short time intervals has been recorded (CIKRT 1972) This variation between rats is considerably less in groups treated in the same series on the same day and such groups are reported in the tables Comparison of the total excretion or the concentrations of mercury between groups may therefore be invalid. The excretion patterns including the effects of the chelating agents are however com 'arable

The chelating agent to be tested was injected intraperitoneally either 3 hours or bout 20 hours after the mercury injection Thus for one group of rats methyl mercuric hloride was injected during the barbiturate narcosis just after the surgical procedures ad been completed and the chelating agent was given 3 hours later For the other roup mercury was injected under slight ether narcosis on the day before the bile ollection period but bile was still collected on the day of the experiment for 3 hours luring barbiturate narcosis before the administration of the chelating agent For both roups the effect of the chelating agent was tested for 3 hours giving a total collection eriod of 6 hours

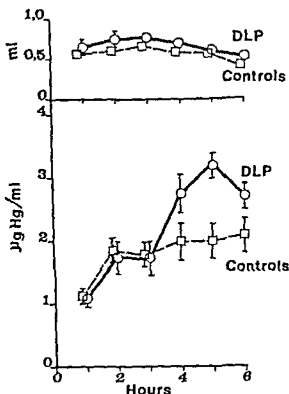
The amount of bile for each time period was determined by weighing and the nercury excretion by counting the collection vials The relative amount of inorganic nercury in bile was determined by the isotopic exchange method (NORSETH & CLARK ON 1970)

Table I shows the chelating agents and doses used in the experiment The treatment vas given with both 3 hours and 20 hours delay periods for all compounds but not for ll doses. Altogether about 100 rats were used divided into groups of from 2 to 8 nimals

Table 1.

Chelating agents tested

mpound	Dose (mg)/rat (200 g)		
mercaptopropanol (BAL)	5		
penicillamine	10	50	100
acetyl DL-penicillamine	5	10	
acetyl-cysteine	250	500	
acetyl homo-cysteine	85	250	500
oacetamide	*10*	*50*	
otic acid	10		
omatic acid	10	50	



Bile volume and mercury concentration in bile of male rats at different time
ıls after cannulation of the bile duct An intravenous injection of 200 µg Hg/rat
/kg) as methyl mercuric chloride was given immediately after cannulation was
d Bile was collected for one hour periods and the results are given as mean ±
When S E M was too small to be drawn outside the circles or squares indicating
alues, it has been omitted in the figure Values are recorded in the figure at the
the corresponding time period The drawn line indicates 7 rats given DL-
lamine (DPL, 50 mg/rat) intraperitoneally after 3 hours The dotted line fr
4 control rats

Results

DL-penicillamine (50 mg/rat) increased the concentration of mercury in bile from 2 μg/ml to 3 μg/ml in male rats when administered 3 hours after the mercury injection (fig. 1) The control rats and the treated group had similar excretion patterns before treatment was given. The mercury concentration increased from 1.1 μg/ml during the first hour to about 1.7 μg/ml during the second and third hour DL-penicillamine increased the mercury concentration at all 3 time intervals tested, the highest concentration being found after the fifth hour The biliary concentration of mercury in the control rats increased from 1.7 μg/ml to 2.1 μg/ml during the last 3 hours of the collection period.

Bile flow in these rats varied from 0.4 ml/hr to 0.8 ml/hr, but DL penicillamine treatment did not affect the flow (fig. 1) The slightly lower bile flow in the control rats is of no significance as the same difference was

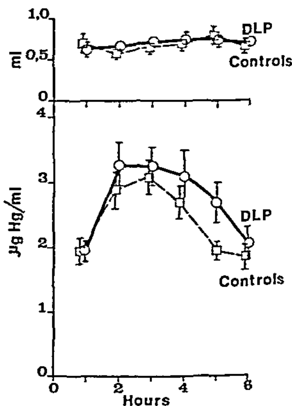


Fig 2. Bile volume and mercury concentration in bile from female rats after cannulation of the bile duct and injection of mercury as indicated in fig. 1 Results are given as in fig. 1 The drawn line indicates 6 rats given DL-penicillamine (DLP 50 mg/rat) intraperitoneally after 3 hours The dotted line indicates 5 control rats

Results

DL-penicillamine (50 mg/rat) increased the concentration of mercury in bile from 2 µg/ml to 3 µg/ml in male rats when administered 3 hours after the mercury injection (fig 1) The control rats and the treated group had similar excretion patterns before treatment was given The mercury concentration increased from 1 1 µg/ml during the first hour to about 1 7 µg/ml during the second and third hour DL-penicillamine increased the mercury concentration at all 3 time intervals tested, the highest concentration being found after the fifth hour The biliary concentration of mercury in the control rats increased from 1 7 µg/ml to 2 1 µg/ml during the last 3 hours of the collection period

Bile flow in these rats varied from 0 4 ml/hr to 0 8 ml/hr, but DL-penicillamine treatment did not affect the flow (fig 1) The slightly lower bile flow in the control rats is of no significance as the same difference was

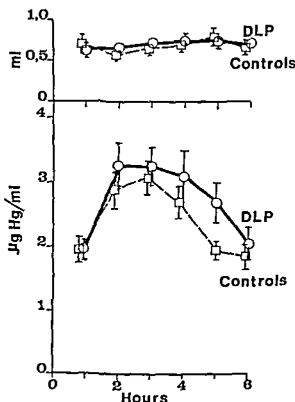


Fig 2 Bile volume and mercury concentration in bile from female rats after cannulation of the bile duct and injection of mercury as indicated in fig 1 Results are given as in fig 1 The drawn line indicates 6 rats given DL-penicillamine (DLP, 50 mg/kg) intraperitoneally after 3 hours The dotted line indicates 5 control rats

found before treatment was given It may have been caused by temperature differences or minor size differences between the groups

Surgical procedures may damage the biliary system and alter the bile flow in the rat The bile flow is also dependent on the position of the tip of the cannula during the collection period In separate experiments it was found that mechanical obstruction of the flow did not alter the concentration of mercury in the bile, but caused a decrease in the total excretion of mercury per time unit This decrease was not compensated for by an increased flow or concentration following the release of the obstruction Most results are therefore presented as concentrations of mercury during a time interval, rather than as total excretion Curves indicating total excretion appear, however, to be similar to the concentration curves

Some chelating agents were given in doses which appeared to be toxic and caused a decrease in bile volume A corresponding increase in mercury concentration was not found in these rats None of the agents tested specifically altered the biliary flow

DL-penicillamine (50 mg/rat) did not increase the biliary concentration of mercury in female rats when administered 3 hours after the mercury injection (fig 2) The mercury concentration increased sharply from 2 μg/ml to 3 μg/ml in both control and in the treated rats from the first to the second hour The highest concentration of mercury in the treated group, 3 3 μg/ml was consequently recorded before treatment was given DL-penicillamine treatment caused a delayed decrease in mercury concentration after 3 hours The concentration in the control rats fell from 3 μg/ml on the third hour to 2 μg/ml on the fifth hour A fall from 3 2 μg/ml to 2 7 μg/ml was recorded for the treated group, the sixth hour there was no difference, and the value, i e 2 μg/ml, was the same as at the first hour Bile volume was not affected by the treatment (fig 2)

Increasing the dose of DL-penicillamine from 50 mg to 100 mg did not change these results Decreasing the mercury dose to 1/10 of the standard dose (0 1 mg/kg) gave a slight increase in mercury concentration after treatment with 100 mg DL-penicillamine, but the increase was not as evident in the female as in the male rats

The mercury concentration in bile 20 hours after the methyl mercuric chloride injection was the same as after 6 hours, i e 2 μg/ml (fig 3) DL-penicillamine given to the female rats at this time period after the mercury injection and 3 hours after the surgical procedure involving cannulation of the bile duct, increased the mercury concentration in the bile from about 1 6 μg/ml to 2 5 μg/ml at the fourth hour and to 2 7 μg/ml at the fifth hour At the last time period recorded there was a fall to 2 4 μg/ml

Dimercaptopropanol (5 mg/rat) had an effect similar to DL-penicillamine

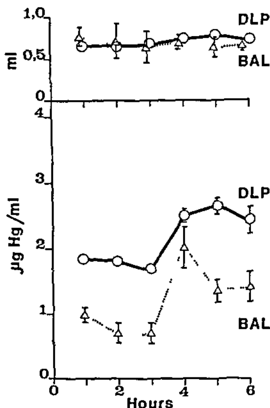


Fig 3 Bile volume and mercury concentration in bile from female rats at different time periods after cannulation of the bile duct An intravenous injection of 200 μg Hg/rat (1 mg/kg) as methyl mercuric chloride was given about 20 hours before cannulation Results are given as in figs 1 and 2 The drawn line indicates 5 rats given DL-penicillamine (DLP, 50 mg/rat) intraperitoneally after 3 hours The dotted line indicates a corresponding injection of dimercaptopropanol (BAL, 5 mg/rat) to 6 rats

e an effect after 20 hours, but not after 3 hours (fig 3) The mercury concentration during the control period was 1 μg/ml for this group, as compared to 2 μg/ml for the DL-penicillamine treated group Differences in the rat groups may explain this difference, though this does not affect the conclusion The bile volume was not significantly different from other groups recorded

Except for occational values probably related to normal variation between the rats, none of the other chelating agents increased the biliary concentration or the total excretion of mercury

Both dimercaptopropanol and DL-penicillamine increased the excretion of organic and inorganic mercury in bile (fig 4) The total excretion per hour after treatment is about twice the excretion per hour in the control period for organic mercury and for inorganic mercury after DL-

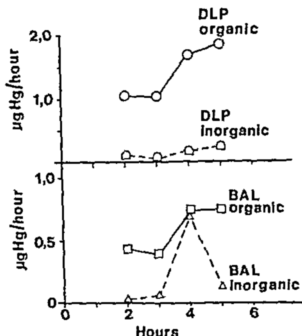


Fig 4 Total biliary excretion of inorganic mercury (dotted line) and organic mercury (drawn line) of rats treated with dimercaptopropanol (BAL) or DL-penicillamine (DLP) as described in fig 3 Bile from all rats in the two groups was pooled The figures are based on duplicate determinations

penicillamine treatment Dimercaptopropanol on the other hand increased the excretion of inorganic mercury by a factor of about 10, but there was a rapid fall after one hour The amount of organic mercury and of inorganic mercury excreted, after DL-penicillamine treatment, remained high for 2 hours which was the time period recorded in this assay For comparing different forms of mercury in the same sample, the absolute amounts are more suitable recorded than the concentrations, as done in this figure

Discussion

DL penicillamine and dimercaptopropanol increase the urinary excretion of mercury after exposure of rats to methyl mercuric hydroxide but the faecal excretion decreases with no net increase (SWENSSON & ULFVARSON 1967) Since these chelating agents increase biliary excretion, they may still be valuable in the treatment of poisoning with methyl mercuric salts An increased excretion of methyl mercuric compounds in mice was recently described after treatment with a resin which interrupts the entero-

circulation of mercury (CLARKSON *et al* 1971) If treatments with chelating agents and resin are combined, this should give valuable results

Neither DL-penicillamine nor dimercaptopropanol decreases the concentration of mercury in the brain after exposure to methyl mercuric hydroxide (SWENSSON & ULFVARSON 1967) The combined treatment described will therefore probably not be effective in the treatment of clinical poisoning with symptoms involving the central nervous system The treatment should be effective, however, in lowering the body load as a result of repeated accumulation and the retention of small doses of methyl mercuric salts, and thus prevent damage to the central nervous system The prevention of damage to foetuses must also be considered Other consequences of methyl mercuric salt exposure as chromosomal breakage or reduced activity of drug metabolising enzymes may also be influenced (SKERFVING *et al* 1970, LUCIER *et al* 1971)

The rat is not the most suitable animal for predicting effects of methylmercury exposure in man because of differences in organ distribution and excretion The biliary excretion of mercury in the rat has been investigated previously, and this animal has therefore been used as a model The biliary excretion of mercury is also found in the mouse and in man (NORSETH 1971, and unpublished results)

The difference between male and female rats in biliary excretion patterns of mercury after the intravenous injection of methyl mercuric chloride is important for the further study of the mechanisms of this excretion process The results may indicate not only a difference in patterns of excretion, but also in total amount excreted Both sexes were not treated within the same series in this experiment Because of variation between groups of rats no [illegible] are thus possible concerning sex differences in total biliary ex-[illegible]tion Sex differences in excretion or retention of mercury related to the intact organism have not been described There is, however, a sex difference in the effect of DL-penicillamine

The time dependent effect of both DL-penicillamine and dimercaptopropanol in female rats indicates some re distribution of mercury within the liver with time The release of inorganic mercury from the intact organomercurial causing re distribution of mercury in the liver has been described (NORSETH & BRENDEFORD 1971) The effect of dimercaptopropanol in the first hour after treatment seems to be directly related to this release of inorganic mercury, not because of re-distribution, but because inorganic mercury is specifically excreted Dimercaptopropanol has no effect on the faecal excretion of mercury either after the administration of inorganic mercury or after methyl mercuric hydroxide (ADAM 1951, SWENSSON & ULFVARSON 1967) The complex of dimercaptopropanol and mercury is probably reabsorbed from the gastro-intestinal tract These results therefore indicate

that a combined treatment with chelating agent and resin should also be tested in the treatment of poisoning with inorganic mercury Total mercury excretion might be increased, but also the re distribution of mercury with increased brain uptake might also be influenced (BERLIN & LEWANDER 1965, MAGOS 1967) The re distribution is a complication of dimercaptopropanol treatment specifically in the treatment of chronic mercury poisoning when the treatment is not immediately life saving

DL penicillamine and dimercaptopropanol are about equally efficient in increasing biliary excretion of the intact organo mercurial Further studies on the distribution and binding of methyl mercuric compounds in the liver are necessary to explain the time dependent effect of these chelating agents on the excretion of methyl mercuric compounds

Screening the chelating agents listed in table 1 revealed none with an effect similar to dimercaptopropanol or DL penicillamine They were, however, not entirely without any effect on the biliary excretion of mercury Thioacetamide for instance decreased the excretion This and other similar compounds may be of value in further investigation on the mechanisms of excretion of mercury compounds or other metals in the bile

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REFERENCES

Adam K R The effects of dithiols on the distribution of mercury in rabbits *Brit J Pharmacol* 1951 6 483–491

Berlin M L.-G Jerksell & G Nordberg Accelerated uptake of mercury by brain caused by 2,3-dimercaptopropanol (BAL) after injection into the mouse of a methylmercuric compound *Acta pharmacol et toxicol* 1965 23 312–320

Berlin M & T Lewander Increased brain uptake of mercury caused by 2,3 d mercaptopropanol (BAL) in mice given mercuric chloride *Acta pharmacol et toxicol* 1965 22 1 7

Cikrt M Biliary excretion of ^{203}Hg ^{64}Cu ^{52}Mn and ^{210}Pb in the rat *Brit J Industr Med* 1972,29 74–80

Clarkson T W H Small & T Norseth The effect of a thiol containing resin on the gastrointestinal absorption and fecal excretion of methylmercury compounds in experimental animals *Fed Proc* 1971 30 543

Lucier G O McDaniel P Brubaker & R Klein Effects of methyl mercury hydroxide on rat liver microsomal enzymes *Chem Biol Interactions* 1971/72 4 265–280

Magos L. Effect of 2 3 d mercaptopropanol (BAL) on urinary excretion and brain content of mercury *Brit J ind Med* 1968 25 152 154

Norseth, T Biotransformation of methyl mercuric salts in the mouse studied by specific determination of inorganic mercury *Acta pharmacol et toxicol* 1971, 29, 375–384

Norseth, T & Margrethe Brendeford Intracellular distribution of inorganic and organic mercury in rat liver after exposure to methylmercury salts *Biochem Pharmacol* 1971, 20, 1101–1107

Norseth, T & T W Clarkson Biotransformation of methylmercury salts in the rat studied by specific determination of inorganic mercury *Biochem Pharmacol* 1970, 19, 2775–2783

Norseth, T & T W Clarkson Intestinal transport of ^{203}Hg labeled methyl mercury chloride Role of biotransformation in rats *Arch Environ Health* 1971, 22, 568–577

Skerfving, S, Kerstin Hansson & J Lindsten Chromosome breakage in humans exposed to methyl mercury through fish consumption *Arch Environ Health* 1970, 21, 133–139

Swensson A & U Ulfvarson Experiments with different antidotes in acute poisoning by different mercury compounds Effects on survival and on distribution and excretion of mercury *Int Arch fur Gewerbepathologie und Gewerbehygiene* 1967, 24, 12–50

Acta pharmacol et toxicol 1973, 32, 11–21

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Studies on the Relationship between the Analgesic Activity of Salicylic Acid and the Brain Catecholamines in Mice

By

Lennart Paalzow

(*Received April 20 1972 Accepted July 20, 1972*)

Abstract The catecholamine content in the mouse brain has been investigated after administration of analgesic doses of salicylic acid Doses of 300 and 450 mg/kg salicylic acid given orally did not produce any significant changes in brain noradrenaline or dopamine levels in comparison with untreated or placebo treated animals After inhibition of the rate limiting step of the catecholamine synthesis salicylic acid in a dose of 300 mg/kg administered orally produced significant acceleration in the decrease of brain noradrenaline induced by the *synthesis inhibitor This acceleration was significantly greater in mice showing* analgesic activity and in these animals a significant acceleration was also found in brain dopamine Furthermore after inhibition of dopamine β hydroxylase with disulfiram salicylic acid produced a significant reduction of the brain nor adrenaline in animals showing analgesic activity No corresponding changes were found in dopamine concentrations The results obtained are similar to those obtained with morphine and it seems possible that salicylates may exert a part of their central analgesic action by interference with noradrenergic or dopamin ergic neurons The investigations also showed that the analgesic test procedure used and the manner of injection did not seem to evoke any stress in the animals sufficient to produce changes in the turnover rate of brain noradrenaline or dopamine

Key words Analgesia – catecholamines – salicyl c acid – stress

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In earlier studies in mice, a good correlation was found between the anal gesic activity and the concentration in the blood of salicylic acid, regardless of whether the animals were given acetylsalicylic acid or sodium salicylate (Ljungberg *et al* 1968) In a following study, Paalzow (1969a) reported that mice given sodium salicylate and showing an analgesic effect had a significantly higher brain concentration of salicylic acid as compared with animals showing no analgesic activity From these results, support was obtained for the view that the salicylates have a central site of action as well as a peripheral one (Winther 1965)

It has been pointed out by several investigators that central adrenergic and

tryptaminergic mechanisms play a role in the analgesic action of morphine (HADLEY & SPENCER 1969, SAARNIVARA 1969, VEDERNIKOV 1969, VEDERNIKOV & AFRIKANOV 1969, LEE & FENNESSY 1970, GARDELLA *et al* 1970, SETHY *et al* 1970, CALCUTT *et al* 1971, PAALZOW & PAALZOW 1971, SPARKES & SPENCER 1971)

The present author therefore thought it of interest to investigate whether the salicylates exerted their central analgesic action by an interference at the catecholamine sites in the brain In the present investigation, the brain catecholamines in mice have been studied in parallel with the registration of analgesic activity after the oral administration of salicylic acid

Materials and Methods

Male N M R I mice weighing 20-22 g were used throughout the study For 16 hours before the test the mice were allowed water *ad libitum* but no food Salicylic acid was given orally as a suspension in 10 % sodium carboxymethylcellulose solution This solution or the physiological saline solution was used as placebo For the electrical stimulation of the animals use was made of two stimulators (Grass Instruments S4) coupled in *train from which electrical square waves are obtained* The voltmeter used was an oscilloscope (Solartron CD 1400)

The analgesic activity was determined by means of a technique described by PAALZOW (1969a & b) In this method, the tail of the mouse is stimulated for 40 msec, using a frequency of the electrical square waves of 125 pulses/sec and a pulse-width of 1 6 msec The electrodes (injection needles No 20) are placed intracutaneously in the middle section of the tail at a distance of about 20 mm apart As specially designed cages are used it is never necessary to remove these electrodes during the course of an experiment The electrodes are applied with the positive pole in the proximal position

A pain reaction is assumed to be produced when the animal responds by squeaking to one of the first three consecutive shocks applied at intervals of one second In each animal the individual pain threshold is determined before administration of the drug and graded in volts This is done by increasing the voltage in logarithmical steps from 4 volts until the animal responds with a squeak After injection of the drug the pain threshold voltage was followed at 10 minute intervals during 1 hour The graded response was expressed as a percentage of the pre treatment pain threshold voltage

The animals were killed by decapitation 60 minutes after salicylic acid or placebo administration and the brain concentration of the catecholamines was determined by a spectrofluorometrical procedure described by SHELLENBERGER & GORDON (1971) After acid extraction subsequent isolation of the catecholamines on alumina and a final oxidation noradrenaline and dopamine were simultaneously determined in a single mouse brain All analyses were run in duplicate and the amounts of the catecholamines were calculated from standard curves

The methyl ester of dl α methyltyrosine (H 44/68) was used as an inhibitor of catecholamine synthesis (ANDÉN *et al* 1966) and H 44/68 was given intraperitoneally in a dose of 250 mg/kg three hours before the administration of salicylic acid or placebo

Disulfiram was given in a dose of 100 mg/kg intraperitoneally 2 hrs before salicylic acid or placebo and was used to block the formation of noradrenaline by the inhibition of dopamine β hydroxylase (GOLDSTEIN & NAKAJIMA 1967)

Table 1

The effect of different treatments on brain noradrenaline and dopamine concentration in mice. Animals tested for analgesic action were killed immediately after six tests performed at 10 minute intervals.
Statistical evaluation was made by analysis of variance

Treatment	Number of animals	Analgesia % Threshold value ± S.E.M	Brain Noradrenaline μg/g ± S.E.M	Brain Dopamine μg/g ± S.E.M
Untreated	18	———	0.321 ± 0.008 *	0.829 ± 0.025 (*)
Test for analgesia	5	100 ± 0	0.338 ± 0.009 *	0.827 ± 0.037 (*)
Test for analgesia + placebo i.v.	5	100 ± 0	0.331 ± 0.014 *	0.780 ± 0.043 (*)

*, (*) } No significant difference between the different groups (P > 0.05)

Results

The effects of the analgesic test procedure on the brain catecholamine levels

A series of experiments was performed to investigate whether the analgesic test procedure and the mode of injection could produce stress in the animals, a condition which can induce changes in the brain catecholamine levels.

After the animals were given placebo intravenously, the pain threshold was followed in each individual mouse at 10 minute intervals and immediately after the test, i.e. 60 minutes after administration, the animals were killed and analyzed for brain noradrenaline and dopamine concentrations.

Table 1 shows that no significant differences in the catecholamine levels were found between the animals run through the test procedure and the untreated animals. However, studies of this type only give very crude information regarding the changes in catecholamine neurons since an alteration in e.g. turnover rates can compensate to varying degrees for changes in monoamine levels. Experiments were therefore undertaken to study the noradrenaline and dopamine brain levels after pre-treatment of the animals with the tyrosine hydroxylase inhibitor H 44/68 or the dopamine β-hydroxylase inhibitor, disulfiram.

The results are given in tables 2 and 3 from which it can be seen that a test procedure performed as in the previous experiment did not significantly influence the noradrenaline or the dopamine brain levels. From these experiments one may conclude that the analgesic test used and the manner of injection did not evoke a state of stress in the animals that was sufficiently strong to produce changes in the turnover rates of brain noradrenaline and dopamine.

Table 2

The effect of different treatments on brain noradrenaline and dopamine concentration in mice Some of the animals were pretreated with H 44/68 4 hours before death The animals tested for analgesic action were killed immediately after six tests performed at 10 minute intervals
Statistical evaluation was made by analysis of variance

Treatment	Number of animals	Analgesia % Threshold value ± S E.M	Brain Noradrenaline µg/g ± S E M	Brain Dopamine µg/g ± S E.M
Untreated	18	———	0.321 ± 0 008	0 829 ± 0 025
H 44/68	5	———	0 220 ± 0 011 *	0 291 ± 0 020 (*)
H 44/68 + test for analgesia	5	106 ± 4	0.219 ± 0 006 *	0.283 ± 0 025 (*
H 44/68 + test for analgesia + placebo i v	23	103 ± 7	0 222 ± 0 004 *	0 285 ± 0 012 (*

* (*) } No significant difference between the different groups (P > 0 05)

Table 3

The effect of different treatments on brain noradrenaline and dopamine concentration in mice Some of the animals were pretreated with disulfiram 3 hours before ı Animals tested for analgesic action were killed immediately after six tests performed at 10 minute intervals
Statistical evaluation was made by analysis of variance

Treatment	Number of animals	Analgesia % Threshold value ± S E M	Brain Noradrenaline µg/g ± S E M	Brain Dopamine µg/g ± S E M
Untreated	18	———	0 321 ± 0 008	0 829 ± 0 025
D sulfiram	5	———	0.228 ± 0 006 *	0 777 ± 0 050 (*)
Disulfiram + test for analgesia	5	93 ± 3	0 217 ± 0 008 *	0 804 ± 0 028 (*)
Disulfiram + test for analgesia + placebo i v	30	94 ± 6	0 229 ± 0 005 *	0 781 ± 0 015 (*

* (*) } No significant difference between the different groups (P > 0 05)

Table 4

The effect of different doses of salicylic acid on brain noradrenaline and dopamine concentration The animals were killed 60 minutes after the administration of salicylic acid

Statistical evaluation was made by analysis of variance

Treatment	Number of animals	Brain Noradrenaline $\mu g/g \pm SEM$	P	Brain Dopamine $\mu g/g \pm SEM$	P
Salicylic acid 300 mg/kg p o	8	0 339 ± 0 008 *	> 0 05	0 831 ± 0 025 (*)	> 0 05
Salicylic acid 450 mg/kg p o	7	0 363 ± 0 008 *		0 856 ± 0 089 (*)	
Placebo p o	8	0 363 ± 0 009	> 0 05	0 775 ± 0 033	> 0 05
Untreated	8	0.340 ± 0 011		0 804 ± 0 040	

*, (*) No significant difference from untreated or placebo treated animals (P > 0 05)

The effects of salicylic acid administration on the brain catecholamine levels

Mice given sodium salicylate or salicylic acid suspension in a dose of 200–300 mg/kg orally show maximal analgesic activity about 40 minutes after administration The maximal effect persists for the following 40 minutes at least (LJUNGBERG *et al* 1968) The investigations of the effects of salicylic acid administration on the brain catecholamine concentrations were therefore performed 60 minutes after oral administration of the drug

In table 4 it can be seen that salicylic acid in doses from 300 mg/kg to 450 mg/kg did not produce any significant changes in brain noradrenaline or dopamine levels in comparison with the untreated or placebo treated animals In the following experiments the pain threshold was followed in each individual mouse at 10 minute intervals during 1 hour after the administration of 300 mg/kg salicylic acid 3 hours before this administration the animals were pretreated with H 44/68 and killed immediately after the test, 60 minutes after salicylic acid administration The control animals were treated in the same way except that they were given a 10 % sodium carboxymethyl cellulose solution orally instead of salicylic acid The animals were divided into two groups One in which analgesia was present and one in which it was absent The range of the graded percentage response was 200–375 % for the first group and 80–100 % for the second group

In this experiment, salicylic acid produced a significant reduction in brain noradrenaline in the test animals as compared to the control animals and

Table 5

The effect of 300 mg/kg salicylic acid on brain noradrenaline and dopamine after inhibition of catecholamine synthesis with H 44/68 given 3 hours before salicylic acid The animals were killed immediately after the test for analgesic activity, 60 minutes after salicylic acid or placebo administration

Statistical evaluation was made by analysis of variance

Treatment	Number of animals	Analgesia % Threshold value ± S E M	P	Brain Noradrenaline µg/g ± S E M	P	Brain Dopamine µg/g ± S E M	P
H 44/68 + salicylic acid 300 mg/kg p o + test for analgesia	5	244 ± 34 **	< 0 01	0 139 ± 0 017 **	< 0 02	0 196 ± 0 022 **	< 0 05
	4	100 ± 0 *		0 192 ± 0 002 **		0 267 ± 0 024 *	
Control H 44/68 + placebo p o + test for analgesia	9	100 ± 0		0 221 ± 0 006		0 289 ± 0 015	

* Not significantly different from control ($P > 0\ 05$)
** Significantly different from control ($P < 0\ 01$)

Table 6

The effect of 300 mg/kg salicylic acid on brain noradrenaline and dopamine after inhibition of dopamine β hydroxylase with disulfiram given 2 hours before salicylic acid The animals were killed immediately after the test for analgesic activity, 60 minutes after salicylic acid or placebo administration
Statistical evaluation was made by analysis of variance.

Treatment	Number of animals	Analgesia % Threshold value ± S E M	P	Brain Noradrenaline µg/g ± S E.M	P	Brain Dopamine µg/g ± S E.M	P
Disulfiram + salicylic acid 300 mg/kg p o + test for analgesia	7	187 ± 37 **		0 160 ± 0 008 ***		0 842 ± 0 026 (*)	
			< 0 001		< 0 001		> 0 05
	18	92 ± 2 *		0 215 ± 0 005 *		0 797 ± 0 018 (*)	
Control Disulfiram + placebo p o + test for analgesia	14	82 ± 4		0 235 ± 0 006		0 825 ± 0 033	

(*) Not s gnificantly different from control (P > 0 05)
* Significantly different from control (P < 0 02)
** Significantly different from control (P < 0 01)
*** Significantly different from control (P < 0 001)

this was significantly greater in animals showing analgesic activity than in animals showing no activity (table 5) The brain dopamine levels were significantly lower only in animals showing analgesic activity as compared to the control animals

In a subsequent experiment the animals were pre-treated with disulfiram 100 mg/kg intraperitoneally instead of H 44/68, 2 hrs before the administration of salicylic acid or placebo As can be seen in table 6, there was a significant reduction in noradrenaline content in mice given salicylic acid as compared to the control animals and this reduction was only seen in mice showing analgesic activity No significant changes were seen in the dopamine concentrations between the different groups (table 6) In these experiments, a significant decrease ($P < 0.001$) in the pain threshold of the control animals treated with disulfiram was also found in comparison with the control animals treated with H 44/68 (table 5)

Discussion

Lim and coworkers (Lim 1960 & 1968, Lim *et al* 1963) have emphasized that the non narcotic, antipyretic analgesics have essentially no central component in their mechanism of analgesic action, and that these drugs exert their action entirely through a peripheral mechanism The receptors for the type of pain which responds to these drugs are said to be chemosensitive Hence acetylsalicylic acid and similar drugs should block the "pain producing substance liberated close to the pain receptor However, Bonnycastle *et al* (1953), Winder (1959) and Paalzow (1969a) have obtained results which support the view that the salicylates have a central site of action as well as a peripheral action Furthermore, Pfeiffer *et al* (1967) reported a dose related change in the quantitative EEG after the administration of acetylsalicylic acid to man The analgesic effect of sodium salicylate has been found to be antagonized by alpha sympathicolytic and catecholamine-depleting drugs (Contreras *et al* 1969) Recently, Dubas & Parker (1971) have reported that sodium salicylate has a selective action in protecting against the nociceptive responses elicited by electrical stimulation of the lateral hypothalamus, but not those evoked by stimulation of the ventral tectum or peripherally In the present investigation, it was found that salicylic acid in doses from 300 to 450 mg/kg did not produce any significant changes in brain noradrenaline or dopamine levels in comparison with untreated or placebo treated animals

In order to look more closely at the relationship between analgesia and brain catecholamines, individual differences among animals were investigated Mice were divided into two groups, one showing and the other not showing

analgesic activity, and the brain catecholamine content was determined at the time of maximal analgesic activity Furthermore by blocking catecholamine synthesis with a potent inhibitor of tyrosine hydroxylase such as the methyl ester of α-methyl tyrosine, more information can be obtained Following the administration of such an inhibitor, the rate of depletion should be proportional to the impulse flow within the neuron, i e the higher the activity, the greater the depletion (ANDÉN *et al* 1966) After pretreatment of the animals with such an inhibitor, salicylic acid in a dose of 300 mg/kg orally produced a significant reduction in brain noradrenaline as compared to the control animals, and this reduction was also significantly greater in animals showing analgesic activity than in animals showing no such activity The brain dopamine concentration was also found to be significantly lower in animals showing analgesic activity, in comparison with the control animals By pretreatment of the mice with disulfiram, which inhibits dopamine hydroxylation, a significant reduction was found in the noradrenaline content in animals given salicylic acid as compared to the control animals and this decrease was furthermore only seen in animals showing analgesia However, in this experiment no change was found in dopamine concentration

Using these two types of inhibitors, salicylic acid was found to decrease the brain noradrenaline concentration to about 33–36 % of that in control animals In studies on the mechanism of central analgesic action of morphine, PAALZOW & PAALZOW (1971) obtained results which support the hypothesis that morphine analgesia is to some extent a result of the libera tion of noradrenaline from the brain stores Thus, about the same results have been obtained for salicylic acid, supporting the view that the salicylates have a central site of action as well as a peripheral one In view of the experiments with the two enzyme inhibitors, it seems possible that the analgesic activity of salicylic acid, as in the case of morphine, is more closely related to an acceleration of the noradrenaline than dopamine turnover However this relationship has to be further evaluated If the salicylates increase the neuronal activity, the greater depletion found after inhibition of the re-synthesis suggest an increase in neuronal activity cannot, be ruled out in the present experiments

CORRODI *et al* (1971) have shown that immobilization stress increases noradrenaline turnover in all parts of the brain and spinal cord and that dopamine turnover in the telencephalon is significantly reduced It is therefore important to investigate whether the test procedure used for estimating the analgesic activity in the animals can influence the brain noradrenaline and dopamine content However, experiments performed after pretreatment of the animals with H 44/68 or disulfiram, or without pretreatment, show that the analgesic test procedure used and the manner of injection did not seem to evoke any stress in the animals sufficiently strong to produce any

changes in the turnover of brain noradrenaline or dopamine The results obtained with salicylic acid therefore seem to be an effect elicited by this acid *per se*

In the experiments in which disulfiram was used, it was found that this drug seems to be able to decrease the pain threshold in the control animals as compared with mice treated with H 44/68 Experiments are now proceeding in our laboratories to evaluate more closely the influence on the normal pain threshold of agents which are known to affect the catecholamine and serotonin levels in the central nervous system

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REFERENCES

Andén, N E, H Corrodi, A Dahlstrom, K Fuxe & T Hökfelt Effects of tyrosine hydroxylase inhibition on the amine levels of central monoamine neurons *Life Science* 1966, 5 561–568

Bonnycastle, D D, L Cook & J Ipsen The action of some analgesic drugs in intact and chronic spinal rats *Acta pharmacol et toxicol* 1953, 9, 332–336

Calcutt C R, N S Doggett & P S J Spencer Modification of the anti nociceptive activity of morphine by centrally administered oubain and dopamine *Psycho pharmacol* (Berl) 1971, 21 111–117

Contreras, E L Tamayo, Y Pincheira & L Quijada Relationship between adrenergic mechanisms and the analgesic effect of sodium salicylate and mefenamic acid *Arch int Pharmacodyn* 1969, 179, 452–458

Corrodi H, K Fuxe, P Lidbrink & L Olson Minor tranquillizers stress and central catecholamine neurons *Brain Research* 1971, 29, 1–16

Dubas T C & J M Parker A central component in the analgesic action of sodium salicylate *Arch int Pharmacodyn* 1971, 194 117–122

Gardella J L J A Izquierdo & I Izquierdo The analgesic action of catecholamines and of pyrogallol *Eur J Pharmacol* 1970 10 87–90

Goldstein M & K Nakajima The effect of disulfiram on catecholamine levels in the brain *J Pharmacol Exp Therap* 1967, 157, 96–102

Hadley, S L & P S J Spencer Analgesic activity after intracerebral injection in the mouse *Brit J Pharmacol* 1969 35, 361p–362p

Lee, J R & M R Fennessy The relationship between morphine analgesia and the levels of biogenic amines in the mouse brain *Eur J Pharmacol* 1970 12 65–70

Lim, R K S Visceral receptors and visceral pain *Ann N Y Acad Sci* 1960 86, 73–89

Lim, R K S, F Guzman K Goto C Braun & D W Rodgers XXX *Federation Proc* 1963, 22 249

Lim R K S XXX In *Pharmacology of pain*, Vol IX p 169 Ed R K S Lim Pergamon Press, London, 1968

Ljungberg S, U Otto & L Paalzow Analgesic activity of acetylsalicylic and salicylic acid and its relation to blood concentration in mice *Acta Pharm Suecica* 1968, 5, 489–500

Paalzow, L An electrical method for estimation of analgesic activity in mice *Acta Pharm Suecica* 1969a, 6, 207–226

Paalzow, L An electrical method for estimation of analgesic activity in mice *Acta Pharm Suecica* 1969b, 6, 193–206

Paalzow, L & G Paalzow Studies on the relationship between morphine analgesia and the brain catecholamines in mice *Acta pharmacol et toxicol* 1971, 30, 104–114

Pfeiffer, C C, L Goldstein & M Hopkins Quantitative electroencephalographic analysis of the effects of aspirin in man *Arch Biol Med Exper* 1967, 4, 10–17

Saarnivaara, L Analgesic activity of some sympathetic drugs and their effect on morphine analgesia in rabbits *Ann Med exp Fenn* 1969, 47, 180–190

Sethy, V H, R J Pradhan, S S Mandrekar & U K Sheth Role of catecholamines in morphine and meperidine analgesia *Indian J Med Res* 1970, 58, 1453–1457

Shellenberger, M K & J H Gordon A rapid, simplified procedure for simultaneous assay of norepinephrine, dopamine and 5 hydroxytryptamine from discrete brain areas *Anal Biochem* 1971, 39, 356–372

Sparkes, C G & P S J Spencer Anti nociceptive activity of morphine after injection of biogenic amines in the cerebral ventricles of the conscious rat *Brit J Pharmacol* 1971, 42, 230–241

Vedernikov, Yu P · Interaction of amphetamine, apomorphine, disulfiram with morphine and the role played by catecholamines in morphine analgesic action *Arch int Pharmacodyn* 1969, 182, 59–64

Vedernikov Yu P & I I Afrikanov On the role of a central adrenergic mechanism in morphine analgesic action *J Pharm Pharmacol* 1969, 21, 845–847

Winder, C V Aspirin and algesimetry *Nature* 1959, 184 494–497

Winther, C A The physiology and pharmacology of pain and its relief In *Analgetics*, p 9–74 Ed G de Stevens, Academic Press, New York and London 1965

Acta pharmacol et toxicol 1973, 32, 22–32

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The Effects of Caffeine and Theophylline on Nociceptive Stimulation in the Rat

By

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Abstract Nociceptive stimulation has been performed in rats with a technique which should allow us to study different pain projection levels within the central nervous system Three different types of thresholds were studied by electrical stimulation and the stimulus responses were qualitatively and quantitatively divided into a) motor response b) vocalisation and c) vocalisation after discharge Caffeine and theophylline were able to decrease the thresholds for motor response and vocalisation in a dose-dependent manner Both drugs were also able to reduce the thresholds for vocalisation after-discharge However, caffeine reduced this response to about 50–60 % of the normal but not in a dose-dependent manner which is contrary to theophylline which in a dose of 100 mg/kg decreased the threshold to as little as 20 per cent of the normal In general maximum effects were obtained 30 minutes after administration and theophylline produced more long lasting effects than caffeine

Key words Caffeine – theophylline – pain

It has been pointed out that adenosine 3',5 monophosphate (cyclic AMP) plays a central role in the mediation of the action of many hormones and is involved in the regulation of metabolic processes There is also experimental evidence that cyclic AMP is involved in the function of the nervous system Theophylline and caffeine are known to inhibit the catabolism of cyclic AMP (for review see Greengard & Costa 1970)

In recent years a number of investigators have suggested that central adrenergic and tryptaminergic mechanisms play a role in the analgesic activity of morphine and related drugs (Clouet & Ratner 1970, Gardella *et al* 1970, Lee & Fennessy 1970, Sethy *et al* 1970, Smith *et al* 1970, Calcutt *et al* 1971, Hitzelmann *et al* 1971, Paalzow & Paalzow 1971, Sparkes & Spencer 1971)

As we have reason to assume that the methylxanthines (e g caffeine and theophylline) are able to affect the central monoamine turnover and syn

thesis (BERKOWITZ *et al* 1970, BERKOWITZ & SPECTOR 1971, WALDECK 1971, CORRODI *et al* 1972) the following experiments were performed to determine whether caffeine or theophylline can influence the threshold responses for nociceptive stimulation in the rat

Materials and Methods

Male Sprague Dawley rats weighing 125–150 g were used throughout the study For 16 hours before the test the rats were allowed water *ad libitum* but no food Caffeine and theophylline were given intraperitoneally as solutions in physiological saline solution

For electrical stimulation of the animals use was made of two stimulators (GRASS INSTRUMENTS S 4) coupled in train from which electrical square waves are obtained The voltmeter was an oscilloscope (SOLARTRON CD1400)

The analgesic activity was determined by means of a technique described by PAALZOW (1969a & b) and later modified by OTTO *et al* (unpublished results) In this method the tail of the rat is stimulated for 1 sec using a frequency of the electrical square waves of 125 pulses/sec and a pulse-width of 1 6 msec The electrodes (injection needles No 20) are placed intra cutaneously in the middle section of the tail at a distance of about 20 mm apart The electrodes are never removed during the course of an experiment and they are applied with the positive pole in the proximal position

In each animal three types of individual thresholds are determined before the administration of the drug and graded in volts By increasing the voltage in logarithmic steps from 0 5 volt the threshold for a *motor response* (tail withdrawal and hind quarters movement) is measured With increasing voltage the animal responds with a squeak (*vocalisation threshold response*) and when the shock is further increased the vocalisation response persists for a short time after cessation of the stimulus (threshold for *vocalisation after discharge*) According to CARROLL & LIM (1960) and HOFFMEISTER & KRONEBERG (1966) the *motor response is a spinal reflex which is subject to facilitating drive from the brain stem* and the vocalisation response which persists after

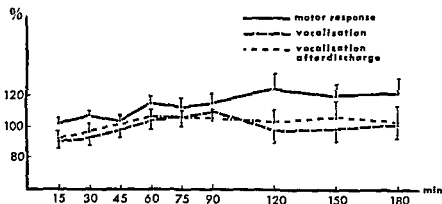


Fig 1 The normal thresholds for motor response vocalisation and vocalisation after discharge followed during 3 hours after intraperitoneal administration of placebo Each point represents the mean ± S E M from 25 rats

transection at a lower pontine level, suggests a reflex pathway through the medulla oblongata

Vocalisation after discharge is most probably relayed at higher levels in the central nervous system and seems to depend on the integrity of a thalamo-hypothalamo-rhinencecephalic system *i e* structures closely associated with the capacity for affective reaction

Table 1

The test of linearity of the normal thresholds for motor response, vocalisation and vocalisation after-discharge after the administration of placebo

MOTOR RESPONSE

	df	Sum of squares	Mean square
Regression	1	9307 94	9307 94
Departure from regression	7	1903 66	271.95
Between tests	8	11211 60	1401 45
Within	241	198774 02	824 79
Total	249	209985 62	

Slope of regression line b = 0 141 ± 0 042
t_b = 3 392 P < 0 001

VOCALISATION DURING STIMULATION

	df	Sum of squares	Mean square
Regression	1	3837 65	3837 65
Departure from regression	7	8379 12	1197 02
Between tests	8	12216 77	1527 10
Within	269	309528 60	1150 66
Total	277	321745 37	

Slope of regression line b = 0 089 ± 0 049
t_b = 1 905 P > 0 05

VOCALISATION AFTER DISCHARGE

	df	Sum of squares	Mean square
Regression	1	3975 28	3975 28
Departure from regression	7	14436 61	2062 37
Between tests	8	18411 89	2301 49
Within	279	298966 72	1071.57
Total	287	317378 61	

Slope of regression line b = 0 089 ± 0 047
t_b=1 905 P > 0 05

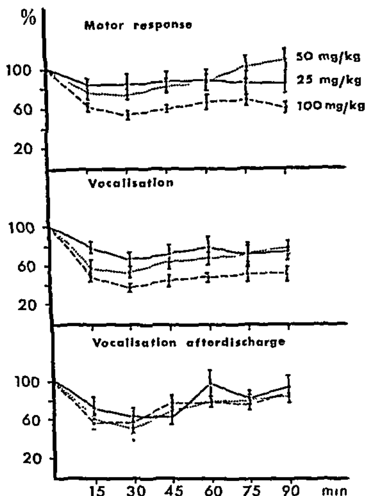


Fig 2 The dose-effect of caffeine on the thresholds for motor response vocalisation and vocalisation after discharge Each po nt represents the mean ± S E M from 10 rats

After injection of the drug these three thresholds are registered at 15 minute intervals and the graded response is expressed as a percentage of the pre treatment threshold voltage

The statistical analyses were performed according to PHARMACOPEA NORD IV (1960)

Results

The effects of drugs on different neurophysiological thresholds

Placebo

EBBECKE (1955) pointed out that there are different pain-projection levels within the central nervous system and it should therefore be appropriate to

use an experimental model which allow the study of the effects on the pain responses relayed through different parts of the central nervous system.

The normal thresholds for motor response, vocalisation and vocalisation after-discharge were studied during 3 hours after the administration of placebo (physiological saline solution) given intraperitoneally in a dose of 5 ml/kg. The results can be seen in fig. 1 and show that during the time period studied the three thresholds remained stable and the slopes of the regression lines of vocalisation and vocalisation after-discharge did not differ significantly from zero (table 1). A slight increase was found in the threshold for motor response during the time period studied. However, this is of minor importance since no significant difference ($P > 0.05$) was found between the different test times.

Caffeine

The threshold determinations were performed on 30 rats divided into groups according to three dose levels and the thresholds were followed during 90 minutes. As can be seen in fig. 2, there was a dose-dependent decrease in the thresholds for motor response and vocalisation during 1 hour after the administration of caffeine. The analysis of regression can be seen in fig. 3 and table 2.

There was also a decrease in the thresholds for vocalisation after discharge to about 50–60 % of the normal but it was not dose dependent. An analysis of regression of the vocalisation after-discharge (fig. 3 and table 2) showed a straight line with a slope which did not differ significantly from zero. Thus, caffeine is able to decrease in a dose dependent manner, the thresholds for motor response and vocalisation to about 40–50 % of the normal threshold in a dose of 100 mg/kg. A maximal decrease was obtained about 30 minutes after injection.

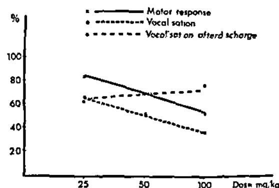


Fig. 3. The log dose response after intraperitoneal administration of caffeine into rats.

Table 2

Test of linearity of the dose response of caffeine on the thresholds for motor response vocalisation and vocalisation after discharge

MOTOR RESPONSE

	df	Sum of squares	Mean square
Regression	1	4774.05	4774.05
Departure from regression	1	144.15	144.15
Between doses	2	4918.20	2459.10
Within	27	13952.50	516.76
Total	29	18870.70	

Slope of regression line b = -15.45 ± 5.02
$t_b = -3.079$ $P < 0.01$

VOCALISATION DURING STIMULATION

	df	Sum of squares	Mean square
Regression	1	4004.45	4004.45
Departure from regression	1	0.15	0.15
Between doses	2	4004.60	2002.30
Within	27	7750.60	287.06
Total	29	11755.20	

Slope of regression line b = -14.15 ± 3.72
$t_b = -3.804$ $P < 0.001$

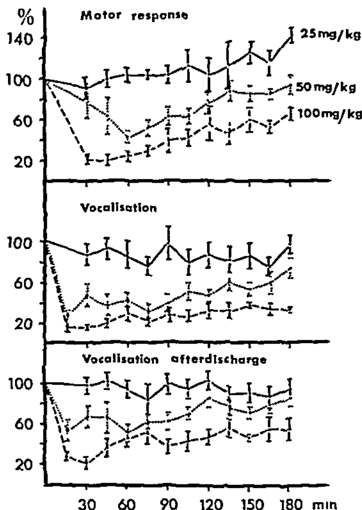
VOCALISATION AFTER DISCHARGE

	df	Sum of squares	Mean square
Regression	1	980.00	980.00
Departure from regression	1	6.67	6.67
Between doses	2	986.67	493.34
Within	27	15979.20	591.82
Total	29	16965.87	

Slope of regression line b = 7.00 ± 5.34
$t_b = 1.310$ $P > 0.2$

Theophylline

The threshold determinations were performed on 20 rats divided up into groups according to three dose levels and the thresholds were followed for 3 hours Theophylline was more potent than caffeine in decreasing the thresholds for motor response, vocalisation and vocalisation after discharge



The dose effect of theophylline on the thresholds for motor response, vocalisation calisation after discharge Each point represents the mean ± S E M from 6 rats.

and in a dose of 100 mg/kg, the thresholds were decreased to about 20 % of the normal (fig 4) Contrary to caffeine, theophylline was even able to decrease the vocalisation after discharge in a dose-dependent manner and the analysis of regression for the different thresholds can be seen in fig 5 and table 3 The effects were more prolonged for theophylline in comparison with caffeine, and a maximal decrease was obtained 30 minutes after the injection, the decrease in the thresholds was still apparent 3 hours after administration of the drug

It was also observed in all experiments that both theophylline and caffeine produced more long lasting vocalisation after discharge as compared to the control animals

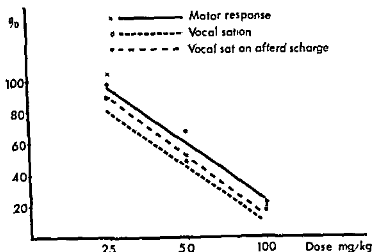


Fig 5 The log dose response lines after intraperitoneal administrat on of theophylline into rats

Discussion

The methylxanthines exhibit a wide range of pharmacological activities (GOODMAN & GILLMAN 1970) We have now found that caffeine and theophylline decrease in a dose dependent manner the thresholds for motor response (suggested spinal reflex) and painful stimuli (vocalisation) Both drugs were able to reduce the threshold for vocalisation after-discharge However, caffeine reduced this response to about 50–60 % of the normal but not in a dose dependent manner This was contrary to theophylline which in a dose of 100 mg/kg gave a decrease as low as about 20 percent of the normal threshold In general, maximum effects were obtained 30 minutes after administration theophylline producing a more prolonged ef fect than caffeine Recently, CONTRERAS *et al* (1972) have reported that theophylline slightly decreases the reaction time in the hot plate analgesic test but not in a dose dependent manner, however The animal test used should allow us to study different pain projection levels within the central nervous system (CARROL & LIM 1960, HOFFMEISTER & KRONEBERG 1966) Thus theophylline seems to differ from caffeine in its ability to decrease the thresholds for vocalisation after discharge a response which is probably relayed at higher levels in the central nervous system and in structures as sociated with the capacity for affective reaction (HOFFMEISTER & KRONEBERG 1966)

During recent years, considerable attention has been paid to the ability of the methylxanthines to affect monoamine turnover and synthesis and also whether these effects are associated with their ability to block the cyclic

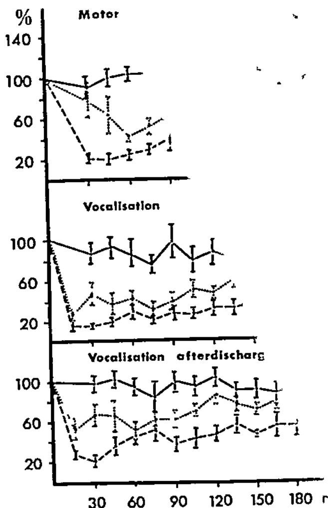


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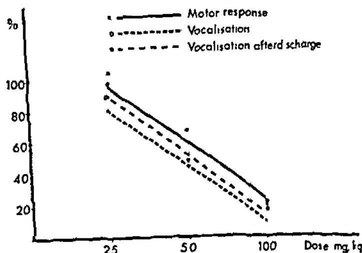


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During recent years, considerable attention has been paid to the ab... of the methylxanthines to affect monoamine turnover and sy... whether these effects are associated with their ability to bl...

Table 3

Test of linearity of the dose response of theophylline on the thresholds for motor response, vocalisation and vocalisation after-discharge

MOTOR RESPONSE

	df	Sum of squares	Mean square
Regression	1	13468 90	13468 90
Departure from regression	1	1080 05	1080 05
Between doses	2	14548 95	7274 48
Within	16	3790 00	236 88
Total	18	18338 95	

Slope of regression line b = −36 70 ± 5 35
t_b = −6 855 P < 0 001

VOCALISATION DURING STIMULATION

	df	Sum of squares	Mean square
Regression	1	18788.56	18788.56
Departure from regression	1	67 84	67 84
Between doses	2	18856 40	9428 20
Within	16	4446.34	277 90
Total	18	23302 74	

Slope of regression line b = −35 96 ± 4 28
t_b = −8 413 P < 0 001

VOCALISATION AFTER DISCHARGE

	df	Sum of squares	Mean square
Regression	1	19907 60	19907 60
Departure from regression	1	150 91	150 91
Between doses	2	20058 51	10029 25
Within	15	5641 10	376 07
Total	17	25699 61	

Slope of regression line b = −38 97 ± 5 26
t_b = −7 415 P < 0 001

nucleotide phosphodiesterase (GREENGARD & COSTA 1970) BERKOWITZ *et al* (1970), BERKOWITZ & SPECTOR (1971), WALDECK (1971) and CORRODI *et al* (1972) found that in rats caffeine in doses of 50–100 mg/kg was able to decrease 5-HT depletion brought about by the tryptophan hydroxylase inhibitor H22/54 but these investigators did not find a significant difference

between the two doses This is in agreement with our own experiments and with the view that 5-HT exerts its activity in some areas of the CNS and could be a mediator of the affective component of the pain reaction HEROLD & CAHN 1968) However, in addition to the decrease found in 5 HT turnover after methylxanthines, it has also been reported that there is a marked decrease in dopamine and an increase in noradrenaline turnover (BERKOWITZ *et al* 1970, WALDECK 1971, CORRODI *et al* 1972) The relationship of these central neuronal changes to the effects found on the thresholds for nociceptive stimulation has to be evaluated

It was interesting to note in our investigation that both caffeine and theophylline markedly increased the duration of the vocalisation after-discharge in the experimental animals as compared to the controls

Acknowledgments

The skilful technical assistance of Miss Lena Abeleen, M Pharm and Mrs Eva Bjorck, M Ph is gratefully acknowledged

REFERENCES

Berkowitz B A, J H Tarver & S Spector Release of norepinephrine in the central nervous system by theophylline and caffeine *Eur J Pharmacol* 1970 10 64–71

Berkowitz, B A & S Spector The effect of caffeine and theophylline on the disposition of brain serotonin in the rat *Eur J Pharmacol* 1971, 16 322 325

Calcutt C R N S Doggett & P S J Spencer Modification of the anti nociceptive activity of morphine by centrally administered oubain and dopamine *Psychopharmacol* (Berl) 1971, 21, 111–117

Carroll M N & R K S Lim Observations on the neuropharmacology of morphine and morphinelike analgesia *Arch int Pharmacodyn* 1960 125 383–403

Clouet D H & M Ratner Catecholamine biosynthesis in brains of rat treated with morphine *Science* 1970 168 854–855

Contreras E S Castillo & L Quijada Effect of drugs that modify 3, 5 Amp concentrations on morphine analgesia *J Pharm Pharmacol* 1972 24, 65–66

Corrodi H, K Fuxe & G Jonsson Effects of caffeine on central monoamine neurons *J Pharm Pharmacol* 1972 24 155 158

Ebbecke U Angeborene Verhaltensweisen des Menschen *Deut Med Wochr* 1955 80 54–57

Gardella J L J A Izquierdo & I Izquierdo The analgesic action of catecholamines and of pyrogallol *Eur J Pharmacol* 1970 10, 87 90

Goodman L S & A Gilman *The pharmacological basis of therapeutics* 1970 4th ed Collier Macmillan London

Greengard P & E Costa (Eds) *Role of cyclic AMP in cell function* 1970 *Raven* Press New York

Herold M & J Cahn The possible role of serotonin in affective component of pain behavioural reaction in the rat In *Pharmacology of pain* 1968 p 87 99 Eds R K S Lim D Armstrong & E G Bardo Pergamon Press Oxford

Hitzemann, R H, H H Loh A K S Ho & E L. Way The effects of acute morphinization on the metabolism of 14C-tyrosine *Proc West Pharmacol Soc* 1971, 14, 91–94
Hoffmeister, F & G Kroneberg Experimental studies in animals on the differentiation of analgesic activity In *Methods in drug evaluation* Eds P Mantegazza & F Piccinini 1966 North Holland Publishing Company, Amsterdam
Lee, J R & M R Fennessy The relationship between morphine analgesia and the levels of biogenic amines in the mouse brain *Eur J Pharmacol* 1970, 12, 65–70
Paalzow, L An electrical method for estimation of analgesic activity in mice *Acta Pharm Suecica* 1969a, 6, 193–206
Paalzow, L. An electrical method for estimation of analgesic activity in mice *Acta Pharm Suecica* 1969b, 6, 207–226
Paalzow, L. & G Paalzow Studies on the relationship between morphine analgesia and the brain catecholamines in mice *Acta pharmacol et toxicol* 1971, 30 104–114
Pharmacopea Nord IV 1960 Apotekarsocietetens forlag Stockholm
Sethy, V H, R J Pradhan, S S Manrekar & U K Sheth Role of catecholamines in morphine and meperidine analgesia *Indian J Med Res* 1970, 58, 1453–1457
Smith, C B, J E Villarreal, J H Bednarczyk & M I Sheldon Tolerance to morphine induced increases in [^{14}C] catecholamine synthesis in mouse brain *Science* 1970, 170, 1106–1107
Sparkes C G & P S J Spencer Anti nociceptive activity of morphine after injection of biogenic amines in the cerebral ventricles of the conscious rat *Brit J Pharmacol* 1971, 42, 230–241
Waldeck, B Some effects of caffeine and aminophylline on the turnover of catechol amines in the brain *J Pharm Pharmacol* 1971, 23, 824–830

Acta pharmacol et toxicol 1973, 32, 33–45

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Inhibition of Histamine Release and Formation of Slow Reacting Substance by Polyphloretin Phosphate

By

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Abstract Polyphloretin phosphate (PPP) a compound with prostaglandin and enzyme inhibitory activities inhibited the release of histamine from rat mast cells induced by compound 48/80 or bee venom but had no effect on the release of histamine produced by chlorpromazine or n-decylamine In perfused cat paws (compound 48/80) and in guinea pig lung tissue (antigen) the histamine release and the formation of slow reacting substance were both inhibited by PPP The blood pressure lowering effect of compound 48/80 in the cat was antagonized by the intravenous administration of PPP Experiments with fractionated (dialysed) PPP showed that the high molecular weight fractions of PPP were more potent that the low molecular weight fractions in inhibiting histamine release from rat mast cells Polyphloroglucinol phosphate a polymer of the same type as PPP but devoid of any prostaglandin inhibitory (IPG) activity, inhibited the release processes in a manner similar to PPP indicating that the IPG activity of PPP is not associated with its capacity to inhibit release It is suggested that the inhibition by PPP of the release processes studied may be due at least partly, to a chemical interaction between inhibitor and releasing agent

Key words Mast cells – histamine release – polyphloretin phosphate

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Polyphloretin phosphate (PPP), a polymerization product of phloretin and phosphorus oxychloride, possesses a strong inhibitory action on several enzymes, particularly hyaluronidase and alkaline phosphatase (DICZFALUSY *et al* 1953) PPP has recently attracted considerable interest due to the fact that it has been shown to antagonize several prostaglandin induced effects selectively both *in vitro* and *in vivo* (BENNETT & EAKINS 1969, EAKINS *et al* 1970, MATHÉ *et al* 1971, MATHÉ *et al* 1972) The prostaglandin antagonizing activity, as evaluated on the gerbil colon, does not seem to be associated with the enzyme inhibitory activity as demonstrated by studies in which PPP fractions, separated by gel chromatography, were used (EAKINS 1971, H FEX B FREDHOLM, B HOGBERG T PERKLEV & S VEIGE, unpublished results)

Recently PPP was found to protect sensitized guinea pigs against the

development of convulsions on exposure to antigen aerosol (STRANDBERG *et al* 1972) This effect of PPP might be due to the demonstrated inhibitory action of PPP on bronchoconstriction induced by prostaglandin $F_{2\alpha}$ or slow reacting substance (SRS) (MATHÉ & STRANDBERG 1971, MATHÉ *et al* 1971 & 1972), since these principles are formed during anaphylaxis in the guinea pig lung (KELLAWAY & TRETHEWIE 1940, PIPER & VANE 1969) However, another mode of action of PPP seems to be relevant in this context, namely the inhibition of histamine release Thus PPP, as well as some other negatively charged polymeric compounds have been reported to inhibit mast cell degranulation and histamine release in tissues of the rat and cat (HÖGBERG & UVNÄS 1957, HÖGBERG *et al* 1957, FERNÖ *et al* 1960, CHAKRAVARTY 1960) and anaphylactic histamine release in guinea pig lung tissue (CHAKRAVARTY 1960)

In the present investigation the inhibitory effect of PPP on histamine release in the rat, cat and guinea pig has been studied with regard to potency, selectivity and mechanism of action In experiments on the cat and guinea pig tissues the effect of PPP on the formation of SRS has also been studied

Furthermore, in order to find out if the histamine release inhibitory activity of PPP is associated with the high or low molecular weight fractions, a study was made using PPP fractions obtained by dialysis

Materials and Methods

Incubation of rat mast cells

Male Sprague Dawley rats (350–400 g) were lightly anaesthetized with ether and bled Nine ml of isotonic salt solution pH 7 0 (NaCl 154 mM KCl, 2.7 mM $CaCl_2$ 0 9 mM) containing 10 % (v/v) Tris HCl buffer (50 mM) was injected into the abdomen and 4 ml into the pleural cavities After lavage of the serous cavities the fluid was withdrawn with a Pasteur pipette The cell suspension was centrifuged at 350 × g for 5 min The supernatant was discarded The residue was suspended in 10 ml of balanced salt solution (see above) and the centrifugation was repeated The cells were then suspended in 20–70 ml of the above salt solution Cells obtained from one rat usually sufficed for 30–40 samples (0 7–1 3 µg of histamine base per sample)

Unless otherwise stated cells were pre-incubated with the inhibitor at 37° for 10 min, the releaser was then added and the incubation continued for a further 10 min The incubation was terminated by placing the tubes in ice water After centrifugation 350 × g at 4° for 10 min, the supernatants were decanted and heated in a boiling water bath for 10 min The residues were similarly heated after the addition of 0 2 ml of 0 1 N HCl The histamine release was calculated as a percentage of the total histamine content of each sample The spontaneous histamine release never exceeded 6 6 % (n = 38) and has been deducted in the data presented

[illegible] tion of guinea pig lung tissue

[illegible] lung tissue obtained from male albino guinea pigs (200–300 g) sensitized to [illegible] n was used [illegible] hylactic reaction The sensitization procedure and prep

aration technique have been described in detail elsewhere (FREDHOLM & STRANDBERG 1969) The lung tissue was suspended (0 1 g/ml) in the above-mentioned salt solution plus glucose (5 6 mM) The tissue was pre-incubated with inhibitors at 37° for 10 min Antigen (1 mg/ml) was then added and the incubation continued for 20 min At the end of the incubation, the tissue and salt solution were immediately separated by filtration After rinsing with salt solution the tissue was suspended in 3 0 ml of 0 1 N HCl Both the filtrates and the tissue suspensions were heated in a boiling water bath and centrifuged Histamine was measured in both filtrates and tissue extracts and the release was calculated as a percentage of the total content of histamine in each sample The spontaneous release never exceeded 2 9 % (n=7) and has been deducted in the data presented SRS-activity was only determined in the filtrates No spontaneous formation of SRS was found

Cat paw perfusion

Cat paws were perfused with an oxygenated salt solution, pH 7 0 (NaCl, 154 mM, KCl, 2 7 mM, $CaCl_2$, 3 6 mM, glucose, 5 6 mM) containing 10 % (v/v) Tris HCl buffer (50 mM) at 27° using temperature controlled chambers and constant rate infusion pumps (1 ml/min) as described earlier (STRANDBERG 1971) One paw of each pair was perfused with releaser and inhibitor, whereas the other paw was perfused with releaser only Inhibitors were added to the perfusion medium 20 min before the addition of the releaser The effluents were collected during 20 min periods in calibrated glass tubes cooled by ice water The perfusates were centrifuged, 350 × g at 4° for 10 min to remove any blood cells present The supernatants were heated in a boiling water bath and centrifuged From each supernatant two 0 5 ml samples were removed for histamine determination The remainder of the supernatant was used for the assay of SRS

Histamine determination

Histamine was determined fluorometrically (SHORE *et al* 1959) using an Aminco-Bowman spectrophotofluorometer The extraction procedure of this method was omitted in the experiments with rat mast cells (FREDHOLM & HAEGERMARK 1967) When albumin was used it was therefore necessary to centrifuge at 900 × g for 10 min, after the addition of HCl, before reading the fluorescence

Assay of SRS

The content of SRS in incubates and perfusates was determined by a three-point biological assay using the isolated guinea pig ileum as described by CHAKRAVARTY (1959) The preparation was suspended in a 5 ml organ bath containing aerated Tyrode solution (37°) with atropine sulphate (1 µg/ml) and mepyramine maleate (1 µg/ml) Longitudinal movements were recorded isotonically on a smoked drum using a frontal writing lever SRS activity is expressed in SRS units referring to a laboratory standard of cat paw SRS (STRANDBERG 1971)

Blood pressure recording

Cats weighing 3–4 kg were anaesthetized with sodium pentobarbital (mebumalum NFN) 25 mg/kg intraperitoneally with intravenous supplement when necessary The trachea was cannulated Heparin (1000 i u /kg) was given Systemic arterial pressure was recorded in the common carotid artery using a Statham pressure transducer and a Grass polygraph

Materials

Compound 48/80, chlorpromazine chloride, a *bee venom preparation* (referred to as "bee venom") obtained by precipitation with picric acid and chromatography on

Amberlite IRC-50, *polyphloroglucinol phosphate* (PGP) *polyphloretin phosphate* (PPP) and fractions of PPP were all kindly supplied by Dr B Hogberg AB Leo Helsingborg Sweden

n Decylamine (puriss) was purchased as the base from Fluka AG Buchs Switzerland *Human serum albumin* was obtained from AB Kabi Stockholm Sweden and lyophilized *egg albumin* from AB Kebo Stockholm Sweden

None of the compounds in the concentrations used interfered with the determination of histamine or SRS

Results

Influence of the inhibitors on processes induced by compound 48/80 and antigen

The inhibitors used, and some of their characteristics, are listed in table 1 The four PPP fractions (F I – F IV) were obtained by dialysis

Preliminary experiments indicated that fraction IV was the most potent with regard to the inhibition of histamine release from rat mast cells (RMC) induced by compound 48/80 Since this fraction had the highest enzyme (hyaluronidase) inhibitory activity and the lowest prostaglandin inhibitory (IPG) activity, as measured on the gerbil colon, another polymer, poly

Table 1

Characteristics of the inhibitors used [a)]

Inhibitor	Relative recovery after dialysis (%) [b)]	Hyaluronidase inhibitory activity [c)]	IPG activity Potency score [d)]	
			$PGF_{2\alpha}$	PGE_1
PPP (Leo 101 i)		1 0	2	2
Fraction I (Leo 1271 a)				
Ist dialysate (0–3 days)	29	0 05	2	2
Fraction II (Leo 1272 a)				
IInd dialysate (3–6 days)	13	0 06	2	2
Fraction III (Leo 1273 a)				
IIIrd dialysate (6–13 days)	11	0 08	3	2
Fraction IV (Leo 1274 a)				
Retentate	47	3 3	1 2	0
PGP (Leo 137 m)		14	0	0

a) from files of AB Leo Helsingborg Sweden

b) 200 g of pure PPP (Leo 101 i) in a volume of 2 l (pH 7 2) was dialysed during 13 days against 3 × 20 l of water

c) determined according to DICZFALUSY *et al* (1953) The relative activities on a weight basis of the inhibitors as compared to the activity of PPP (1 0) are presented

d) as evaluated on the gerbil colon

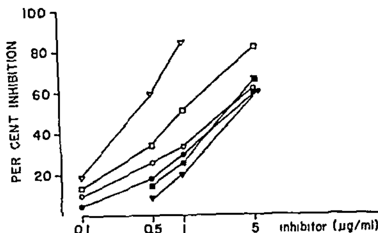


Fig. 1. Inhibition of compound 48/80 (1µg/ml) induced histamine release from rat mast cells by PPP (○—○) FI (▼—▼) FII (■—■) FIII (●—●) FIV (□—□) and PGP (▽—▽). Each point represents the mean of 6 experiments. Standard error was < 6 % in all the experiments.

phloroglucinol phosphate (PGP), with a still higher enzyme inhibitory capacity and devoid of any IPG activity was included in the study.

Rat mast cells. As shown in fig. 1, compound 48/80 induced histamine release from RMC was inhibited by all the inhibitors used. The order of potency was PGP, F IV, PPP, F III, F II and F I. The concentration required to produce 50 % inhibition of the release caused by 1 ug/ml of 48/80 (ID 50) was from 0.35 to 2.8 µg/ml. Thus the present findings show that both the low and high molecular weight fractions of PPP exert an inhibitory action on compound 48/80 induced histamine release from RMC and that on a weight basis the high molecular weight fractions are more potent in this respect. The IPG activity of the fractions does not seem to be related to the histamine release inhibition since PGP, which is devoid of IPG-activity, was the most active compound.

Fig. 2 illustrates the dose effect relations when RMC were incubated with various doses of compound 48/80 in the presence or absence of the above ID 50 concentrations of PGP and F IV. It can be seen that, regardless of the inhibitor, the antagonism fulfills the criteria for competitive action, i.e. parallel shifts of the dose-effect plots without any change in the maximal effect of the agonist. In a double-reciprocal plot of the values, approximately the same intercepts on the y axis were found. These findings indicate that in this system the inhibitors used have a similar mechanism of action.

Cat paw and guinea pig lung tissue. So far, most inhibitors of compound 48/80 induced histamine release in RMC have also been found to inhibit histamine release and the formation of SRS produced by the same agent

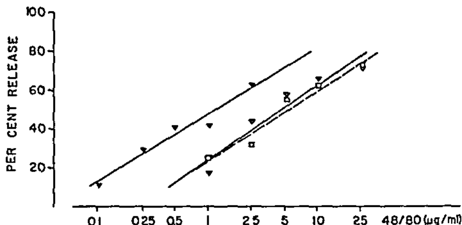


Fig 2 Dose-effect relations for histamine release from rat mast cells induced by com pound 48/80 in the absence (▼—▼) or presence of 0 35 µg/ml of PGP (▽—▽) or 1 µg/ml of FIV (□---□) The regression lines are calculated according to the least square method

in the cat paw (STRANDBERG 1971) and by antigen in guinea pig lung tissue (for references see MONGAR & SCHILD 1962) Therefore the effects of the present inhibitors were also studied in these tissues Due to the limited supply of fractionated PPP, only the effects of PPP and PGP were studied in the cat paw whereas in the experiments on guinea pig lung tissue all the inhibitors were used It was found that in the cat paw, both PPP and PGP inhibited the histamine release and the formation of SRS in a dose-dependent manner (fig 3) PGP was also somewhat more potent than PPP in this system In contrast PGP, in the concentration studied, had the lowest inhibitory effect on the anaphylactic release processes in sensitized guinea pig lung tissue (table 2) Apart from the lack of effect of F III on the antigen induced formation of SRS, all the fractions had about the same potency as PPP with regard to the inhibition of the anaphylactic release processes

Influence of protein on the inhibition

The inhibitory action of PPP on hyaluronidase *in vitro* is reduced *in the presence of albumin or serum* (*FEX, personal communication*) In the present study it was found that the presence of human serum albumin in the incubation medium reduced the inhibitory effect of both PPP and PGP on compound 48/80 evoked histamine release from RMC (fig 4) Thus 500 µg/ml of albumin reduced the inhibitory effect of PPP (2 7 µg/ml) and PGP (0 35 µg/ml) by about 50 and 85 % respectively. Increasing the dose of PPP to 10 µg/ml (not shown in the figure) almost abolished the depressant effect of albumin As has been reported earlier (UVNÄS & THON 1959), the releasing effect of compound 48/80 was higher when albumin

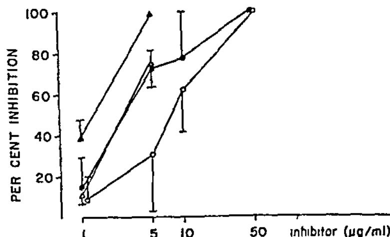


Fig 3 Inhibition of compound 48/80 (1 µg/ml) induced histamine release (open symbols) and formation of SRS (closed symbols) in the cat paw by PPP (circles) and PGP (triangles) The results are expressed as percentages of the output of histamine and SRS from control paws perfused with compound 48/80 alone Means and standard errors of 4 experiments

was present in the medium than when it was not Thus compound 48/80 (1 µg/ml) released 52 2 ± 2 7 % (mean ± S E M , n = 12) of the total histamine in the absence of albumin, whereas 66 4 ± 1 1 % (n=12) was released in the presence of 50 µg/ml of albumin ($P < 0\ 001$)

Influence of PPP and PGP on the effect of other histamine releasers

Since both PPP and PGP are acidic in nature the question arose whether the inhibitory action of these substances on the processes induced by the basic polymer compound 48/80 might be due to chemical interactions To investigate this possibility, the inhibitory capacity of PPP and PGP on histamine release in RMC induced by other liberators, was studied and

Table 2

Inhibition of antigen induced release of histamine and formation of SRS in guinea pig lung tissue by PPP FI – FIV and PGP The concentration of inhibitor was 100 µg/ml Means and standard errors of 5 experiments

	Inhibition in per cent of control					
	PPP	FI	FII	FIII	FIV	PGP
Histamine	59 0 ± 14 2	61.4 ± 10 1	60 8 ± 14.3	60 2 ± 14 0	55 4 ± 7 0	32 2 + 9 1
SRS	59.5 ± 12 0	61 6 ± 10 2	82 3 ± 3 1	4 4 ± 10 1	73 7 ± 14 1	40 6 ± 18 2

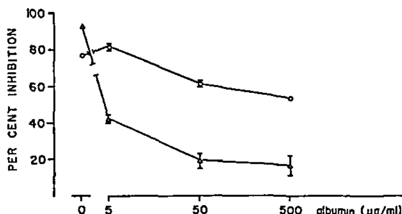


Fig 4 Effect of human serum albumin on the inhibition of compound 48/80 (1 µg/ml) induced histamine release from rat mast cells produced by 2 7 µg/ml of PPP (O—O) and 0.35 µg/ml of PGP (Δ—Δ) Means and standard errors of 6 experiments

compared with the effect on compound 48/80 evoked release The releasers selected were bee venom, chlorpromazine and n-decylamine The effect of different concentrations of inhibitor on the ED50 of these agents was studied As shown in table 3, PPP and PGP inhibited compound 48/80 and bee venom induced histamine release, whereas in the concentrations used no inhibitory effect on the release produced by chlorpromazine and n decylamine was found On the contrary, PGP (1 µg/ml) significantly ($P < 0\ 01$) promoted the histamine releasing effect of both chlorpromazine and n decylamine

Influence of pre incubation

In the above experiments the inhibitors were added to the incubation media before the addition of the releaser In view of a possible chemical

Table 3

Influence of PPP and PGP on the histamine release from rat mast cells produced by compound 48/80 (1 µg/ml) bee venom (2 5 µg/ml) chlorpromazine (23.5 µg/ml) and n-decylamine (20 µg/ml) Means and standard errors of 8 experiments

Inhibitor	Inhibition in per cent of control			
	compound 48/80	bee venom	chlorpromazine	n-decylamine
PPP, 10 µg/ml	NS	68 6 ± 1 9	−0.5 ± 1 4	− 2 9 ± 2.3
PGP, 1 µg/ml	89 6 ± 2 0	89 2 ± 2 9	−8 2 ± 2 3	−14 1 ± 3 9
PGP, 10 µg/ml	NS	81 4 ± 4 0	−0 8 ± 2 0	− 9 2 ± 4 2

NS = not studied

Table 4

Influence of pre incubation time on the inhibitory effect of PPP (2 7 μg/ml) and PGP (0 35 μg/ml) on compound 48/80 (1 μg/ml) induced histamine release from rat mast cells Means and standard errors of 8 experiments

Inhibitor	Time interval between additions of inhibitor and releaser (min)		
	0	0.5	10
PPP	96.3 ± 0 4	89 4 ± 0 7	88 2± 0 7
PGP	99 8 ± 0 4	80 6 ± 0 7	75 0 ± 1 9

interaction between histamine releaser and inhibitor it was thought that such an action would be enhanced if both agents were administered at the same time Table 4 shows the results when RMC were incubated with compound 48/80 (1 μg/ml) and PPP and PGP were added 10 min before, half a minute before, or at the same time as the releaser It was found that the inhibition of histamine release produced by PPP and PGP was significantly ($P < 0\,001$) higher when releaser and inhibitor were mixed and administered than when the inhibitor was added in advance

Effect of PPP in vivo

The effect of PPP on the blood pressure lowering effect of compound 48/80 was investigated in the cat PPP was infused intravenously during a 5 min period and compound 48/80 was administered via the same route 10 min later The intravenous *injection of 10 μg/kg of compound 48/80* produced a sustained fall in blood pressure (2 cats) Pre treatment with 5 mg/kg of PPP was found to inhibit completely the depressor action of 25 μg/kg of compound 48/80 whereas it had no effect on the blood pressure lowering effect of histamine (2 cats) However, subsequent administration of compound 48/80, 50 μg/kg, resulted in a pronounced fall in blood pressure In another cat given 40 mg/kg of PPP the depressor action of 250 μg/kg of compound 48/80 was almost totally blocked Here too the subsequent administration of a higher dose of compound 48/80, 500 μg/ml, overcame the blockade The experiments described are illustrated in fig 5

Discussion

The present results show that polyphloretin phosphate (PPP) can inhibit histamine release induced by compound 48/80, bee venom and antigen In the tissues studied, these agents initiate release process(es) by activating

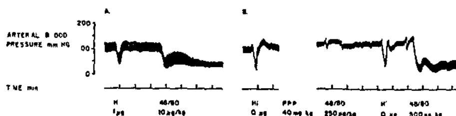


Fig 5 Inhibitory effect of PPP on the blood pressure lowering effect of compound 48/80 in the cat Two different experiments Experiment A show the depressor effects of histamine (Hi) and compound 48/80 in an anaesthetized cat not given PPP Experiment B shows the effect of these compounds after pretreatment with PPP Time interval between PPP and compound 48/80 was 10 min

enzymatic mechanisms (c f MONGAR & SCHILD 1962, UVNÄS 1963, FREDHOLM & HAEGERMARK 1967, STRANDBERG 1971) On the other hand, PPP had no effect on histamine release from rat mast cells induced by chlorpromazine or n decylamine This might be taken as evidence for a selective effect of PPP, since these compounds are known to evoke histamine release from rat mast cells by mechanisms which differ from those of the other releasers used (MORAN *et al* 1962, BLOOM & HAEGERMARK 1967, FRISK HOLMBERG 1972)

From the present results the mode of action of PPP as an inhibitor of histamine release cannot be stated with certainty The effect does not seem to be associated with the prostaglandin inhibitory (IPG) activity of the polymer since there was no correlation between this activity of the fractions and their capacity to inhibit histamine release Further support for this view was provided by the findings that polyphloroglucinol phosphate (PGP) which is devoid of IPG activity as evaluated on the gerbil colon inhibited both compound 48/80 induced and anaphylactic histamine release However, since PPP is negatively charged, it seems likely that its action is at least in part due to chemical interaction with the releaser It is known that electrically charged polymers tend to interact more easily with other polymers e g compound 48/80, than with simpler molecules e g chlorpromazine Furthermore, it was found that the inhibition of compound 48/80 induced histamine release from rat mast cells was significantly higher when the releaser and inhibitor were mixed and added to the incubation medium than when the cells were pretreated with the inhibitor A similar effect of the pre incubation time on the inhibitory action of disodium cromoglycate on compound 48/80 induced histamine release has been reported (ORR *et al* 1971) It is also possible that the inhibition of anaphylactic histamine release by PPP in guinea pig lung tissue is a result of a chemical interaction The fact that the addition of albumin to the incubation medium significantly reduced the inhibitory activity of PPP on compound

48/80 induced histamine release from rat mast cells indicates that PPP binds to albumin

PPP and PGP antagonized not only histamine release but also the formation of slow reacting substance (SRS) induced by compound 48/80 and antigen This suggests that if the inhibitors act on the release process *per se* it must be at an early step since the release of histamine and the formation of SRS produced by these agents are thought to be triggered off by a common mechanism, after which they proceed independently (CHAKRAVARTY & UVNÄS 1960, STRANDBERG 1971)

Fractions of PPP, obtained by dialysis were used in order to establish whether the inhibitory effect of PPP in the histamine release process was confined to the low or high molecular weight components of PPP and associated with any of the known properties of PPP, enzyme inhibition and IPG activity However, all fractions of PPP were about equally potent in inhibiting the anaphylactic release processes despite the fact that they differed markedly in IPG activity and in enzyme inhibitory properties In rat mast cells fraction IV was most potent, indicating that in this system the inhibitory activity of the high molecular weight enzyme inhibitory fractions of PPP was higher than that of the low molecular weight fractions

It was found that intravenously administered PPP counteracted the blood pressure lowering effect of intravenously injected compound 48/80 The depressor action of compound 48/80 has mainly been attributed to its histamine releasing effect (PATON 1951, ROSÉN *et al* 1957) The possibility that PPP can also block histamine release *in vivo* is of particular importance since the intravenous administration of PPP has been shown to protect sensitized guinea pigs against the development of convulsions on exposure to antigen aerosol (STRANDBERG *et al* 1972) Histamine is known to be one of the main mediators of anaphylactic bronchoconstriction in the guinea pig (c f COLLIER & JAMES 1970) It is thus possible that the protective effect of PPP seen in anaphylaxis might, at least partly, be ascribed to the histamine release inhibitory property of the compound The antagonizing action of PPP on bronchoconstriction (MATHÉ & STRANDBERG 1971, MATHE *et al* 1971 & 1972) elicited by two other postulated mediators, namely prostaglandin $F_{2\alpha}$ and SRS might also contribute to the beneficial effect Further work will explore whether any of these properties is of significance for the ameliorating effect of PPP in anaphylaxis in the guinea pig

Acknowledgements

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REFERENCES

Beitch, B R & K E Eakins The effects of prostaglandins on the intraocular pressure of the rabbit *Brit J Pharmacol* 1969, 37, 158–167

Bloom G & Ö Haegermark Studies on morphological changes and histamine release induced by bee venom n decylamine and hypotonic solutions in rat peritoneal mast cells *Acta physiol scand* 1967, 71, 257–269

Chakravarty N A method for the assay of "Slow reacting substance" *Acta physiol scand* 1959, 46, 298–313

Chakravarty, N The mechanism of histamine release in anaphylactic reaction in guinea pig and rat *Acta physiol scand* 1960, 48, 133–145

Chakravarty, N & B Uvnas Histamine and a lipid soluble smooth muscle stimulating principle (SRS) in anaphylactic reaction *Acta physiol scand* 1960, 48, 302–314

Collier, H O J & G W L James Humoral factors in airway function with particular reference to anaphylaxis in the guinea pig In *Airway dynamics* Ed C C Thomas Springfield, 1970 239–252

Diczfalusy, E., O Ferno, H Fex, B Hogberg T Linderot & T H Rosenberg Synthetic high molecular weight enzyme inhibitors *Acta chem scand* 1953, 7, 913–920

Eakins, K E Prostaglandin antagonism by polymeric phosphates of phloretin and related compounds *Ann New York Acad Sci* 1971 180 386–395

Eakins, K E, S M M Karim & J D Miller Antagonism of some smooth muscle actions of prostaglandins by polyphloretin phosphate *Brit J Pharmacol* 1970 39, 556–563

Ferno O B Hogberg & B Uvnas The inhibitory action of a polycarboxylic acid on the disruption of mast cells and liberation of histamine produced by compound 48/80 ascaris extract, antigen – antibody reaction or phospholipase A *Acta pharmacol et toxicol* 1960 17, 18–26

Fredholm B & Ö Haegermark Histamine release from rat mast cells induced by a mast cell degranulating fraction in bee venom *Acta physiol scand* 1967, 69, 304–312

Fredholm, B & K Strandberg Release of histamine and formation of smooth muscle stimulating principles in guinea pig lung tissue induced by antigen and bee venom phosphatidase A *Acta physiol scand* 1969, 76 446–457

Frisk Holmberg M Drug induced changes in the release and uptake of biogenic amines *Acta physiol scand* 1972, 84, Suppl 376

Hogberg B & B Uvnas The mechanism of disruption of mast cells produced by compound 48/80 *Acta physiol scand* 1957, 41, 345–369

Hogberg B, G Sudow I L Thon & B Uvnas The inhibitory action of a compound obtained from hip seeds (HSC) on the release of histamine and the disruption of mast cells produced by compound 48/80 and extracts from jelly fish (Cyanea capillata) and eelworm of swine (Ascaris lumbricoides) *Acta physiol scand* 1957, 38, 265–274

Kellaway, C H & E R Trethewie The liberation of a slow reacting smooth muscle stimulating substance in anaphylaxis *Quart J Exp Physiol* 1940, 30, 121–145

Mathé A A & K Strandberg Antagonism of slow reacting substance by polyphloretin phosphate on isolated human bronchi *Acta physiol scand* 1971, 82, 460–465

Mathé, A A, K Strandberg & A Åstrom Blockade by polyphloretin phosphate of the prostaglandin $F_{2\alpha}$ action on isolated human bronchi *Nature New Biol* 1971, 230, 215–216

Mathé, A A, K Strandberg & B Fredholm Antagonism of prostaglandin $F_{2\alpha}$ induced bronchoconstriction and blood pressure changes by polyphloretin phosphate in the guinea pig and cat *J Pharm Pharmacol* 1972, 24 378–382

Mongar, J L & H O Schild Cellular mechanisms in anaphylaxis *Physiol Rev* 1962, 42 226–270

Moran, N, B Uvnas & B Westerholm Release of 5 hydroxytryptamine and histamine from rat mast cells *Acta physiol scand* 1962, 56 26–41

Orr T S C, D E Hall, J H G William & J S G Cox The effect of disodium cromoglycate on the release of histamine and degranulation of rat mast cells induced by compound 48/80 *Life Sci* 1971, 10 805–812.

Paton, W D M Compound 48/80 A potent histamine liberator *Brit J Pharmacol* 1951, 6 499–508

Piper, P J & J R Vane Release of additional factors in anaphylaxis and its antagonism by anti inflammatory drugs *Nature (Lond)* 1969, 223, 29–35

Rosén, A, P Strandberg & B Uvnas Vascular effects of moderate doses of histamine and a histamine liberator (compound 48/80) *Acta pharmacol et toxicol* 1957, 13 267–276

Shore, P A, A Burkhalter & V H Cohn jr A method for the fluorometric assay of histamine in tissues *J Pharmacol exp Therap* 1959, 127, 182–186

Strandberg K. Release of histamine and formation of slow reacting substance in the cat paw induced by compound 48/80 *Acta physiol scand* 1971, 82 500–511

Strandberg K., A A Mathé & B Freedholm Protective effect of polyphloretin phosphate in anaphylaxis in the guinea pig *Life Sci* 1972, 11, 701–702

Uvnas B Mechanism of histamine release in mast cells *Ann New York Sci* 1963, 103, 278–283

Uvnas, B & I L. Thon Isolation of "biologically intact' mast cells *Exp Cell Res* 1959, 18, 512–520

Acta pharmacol et toxicol 1973, 32, 46–64

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The Fate of Atropine in the Puppy

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Abstract The fate of atropine in newborn, 3 and 6 weeks old, 3 months old and adult dogs has been investigated with the tritium labelled drug After subcutaneous injection (0.5 mg/kg) maximal plasma concentrations of radioactivity were obtained after 0 5 hr in the two oldest groups and after 1–1.5 hrs in the three youngest groups The plasma half life of radioactivity was considerably longer in the newborn animals than in the other groups (11.5 hrs as against 3 5–5.5 hrs) In the three youngest age groups, the urinary excretion of radioactivity was about half that in the adult animals who excreted about 40 per cent of the injected radioactivity in 4 hrs Between 50–90 per cent of the radioactivity in the urine was recovered as the unchanged drug in all age groups in the 2 to 4 hrs interval There were no consistent differences in the pattern of metabolites in the urine between the different age groups The concentration of radioactivity in the liver, kidney submandibular gland, heart skeletal muscle and brain was investigated at 0 5 1, 2, 4 and 8 hrs after injection (0 5, 2 and 8 hrs for adult animals) In general, there were no significant differences in organ distribution between the age groups The radioactivity in the brain consisted exclusively of the unchanged drug The rate of penetration into the brain and the maximal brain concentrations of the drug showed no significant changes with age Within the hemispheres, a fall in the concentration of atropine from the cerebral cortex to the lateral ventricles was found in all age groups The binding of atropine to plasma proteins was about 18 per cent over a wide range of concentrations No difference in plasma binding *in vivo* was found between the different age groups

Key words Atropine – pharmacokinetics – age factors – dogs

Species differences in the toxicity of atropine are generally claimed to be due to differences in the metabolism of the drug Lethal doses of atropine are lower in dogs than in rats and mice (WILLBERG 1914, OBATA 1931, KANDA 1941, GOLDENTHAL 1971) and dogs have a slower metabolism of atropine than mice and rats and excrete a larger part of the drug in the unchanged form (ALBANUS *et al* 1968a, GOSSELIN *et al* 1955, GABOUREL & GOSSELIN 1958, KALSER *et al* 1957, WERNER & SCHMIDT 1968) There are some reports

in the literature of a change in sensitivity to anticholinergic drugs during development Increased as well as decreased sensitivity has been described, evidently depending on the species used In rats and mice, atropine is less toxic in adult animals than in the newborn (OBATA 1931, KANDA 1941, GOLDENTHAL 1971), while the opposite relationship has been reported for the dog (10 weeks-old puppies as against adult dogs, WILLBERG 1914) In the rat this difference in toxicity has been claimed to depend on a slower metabolism of the drug in the newborn animals, since no difference in toxicity was found between 10 days old and adult rats after intravenous administration (KANDA 1941)

In order to evaluate the hypothesis of a pharmacokinetic explanation for the toxicity of the drug the distribution, metabolism and excretion of atropine were studied in dogs of different ages, ranging from newborn to adult

Methods

Thirty beagle puppies of both sexes from 17 litters were used at the following age intervals newborn (1–5 days) three weeks (18–25 days) 6 weeks (40–50 days) 3 months (85–100 days) In addition 3 adult dogs (over 300 days) were used During the experiments the animals were kept in boxes (newborn and 3 weeks) or in cages of 1 m² The box temperature was kept at 24–27° for the newborn dogs otherwise at room temperature Dogs in the three youngest groups were kept with their mothers until immediately before the experiments Newborn animals were fed with milk once every second hour The labelled atropine generally tritiated 434 mci/mmol (Radiochemical Centre Amersham England) with the amount of carrier atropine sulphate required to give a dose of 0 5 mg/kg calculated as atropine sulphate was given subcutaneously into the neck or intravenously in 1 ml/kg to the two youngest groups and in 0 1 ml/kg of distilled water to the older dogs The injected radioactivity varied between 50 and 250 µci per kg bodyweight depending on the type of experiment

Blood samples (0 2 ml) were drawn with a heparinized syringe through catheters in the saphenous vein inserted about one hour before the experiments with the animals under Halothane-nitrous oxide anaesthesia In the newborn dogs a catheter was instead inserted under pentobarbital anaesthesia (10–20 mg/kg intraperitoneally) into the jugular vein before the experiment This group of animals usually remained sedated during the experiments while the other groups recovered completely before the experiments In carcasses blood was obtained by cardiac puncture immediately after sacrifice Urine samples were obtained through catheters (Feeding tube 5 Ch Eshmann England) inserted into the bladder via the urethra at the same time as the venous catheter For technical reasons only male dogs were used for urine sampling In the newborn dogs only animals killed at the end of the collection period were used for calculation of total urinary excretion since efficient rinsing of the bladder could not be performed

When organ concentrations of radioactivity were determined the animals were killed by an intravenous or intracardiac injection of pentobarbital (mebumalum NFN) and immediately dissected Different brain parts were obtained by dissection of transverse sections of the brain Ventriculocortical gradients in the hemisphere were obtained by cutting out a piece of tissue reaching from the middle part of the body of the lateral ventricle to the parietal cortex and then dividing it by three equidistant transverse

sections (fig 9) Macroscopically, the three tissue pieces closest to the ventricles consisted mainly of white matter Cerebrospinal fluid (CSF, 0 2 ml) was obtained by puncture of the cisterna magna in the carcasses

Assay of radioactivity in organs was done after oxygen combustion as described elsewhere (ERIKSSON & WINBLADH 1971) Bile (0 01 ml) urine (0 01 ml) and plasma (0 1 ml) were directly mixed with the scintillation solvent (Triton X 100 500 ml toluene 500 ml omnifluor® 4 g NEN Corp, Boston, USA) and assayed by liquid scintillation counting (CPM 2000 Beckman) In the case of plasma, no differences were noted between this direct method and the extraction or combustion methods mentioned below When not otherwise stated, results are given as $\left(\frac{\text{d p m /g tissue wet weight}}{\text{d p m /g injected}}\right) \times 100$ Since the tissue was dried to constant weight at room temperature or at 50° before combustion the tissue water content could be estimated simultaneously When investigating metabolites in tissues and plasma, acid ethanol extracts were made according to ALBANUS *et al* (1968a) Before chromatography the extracts were when necessary concentrated 2–10 times by vacuum evaporation 10–40 µl of extract, bile or urine was submitted to paper chromatography as described elsewhere (ALBANUS *et al* 1968a) However concentration of the extracts caused increased tailing and also decreased the RF value for atropine and the metabolites In extracts from liver concentrated 10 times and in bile, the RF values for atropine were 0.5–0 65 while the corresponding values in aqueous solution was 0 72–0 80 For this reason labelled atropine was always added to an aliquot of the extract and chromatographed in parallel After localization of the different peaks with a Packard strip scanner, their radioactive content was determined by combustion of transverse sections of the paper strips When the activity on the chromatogram was too low for radio scanning the paper strips were cut into 3 cm pieces and combusted The mean recovery from the chromatographic strips was 89 ± S E M 6 per cent of the spotted radioactivity in the extracts. Determination of plasma protein binding was performed by ultra filtration with a miniturization of the method of BORGÅ *et al* (1969) Calculation of half life of total plasma radioactivity was made by fitting a line according to the least square method to all values from one age group on curve parts which appeared reasonably linear in a semilogarithmic plot with the aid of a FOCAL programme run on a PDP 8 computer (Digital Equipment Corp, USA) Statistical evaluation of differences between the different age groups was done with a t test according to OSTLE (1954)

Results

Plasma concentrations of radioactivity

After the subcutaneous injection of 0 5 mg/kg, the maximal concentration is reached later in the three youngest groups (60–90 min) than in the two oldest groups (30 min , fig 1) The maximal concentrations are similar in all age groups except in the six weeks old puppies, for whom a 45 per cent higher value was found From 1 to 1 5 hrs after the injection, the elimination of radioactivity from the plasma is linear in a semilogarithmic plot, except in the adult dogs, where the elimination curve appears to be composed of an initial fast and a second slower component After the intravenous injec

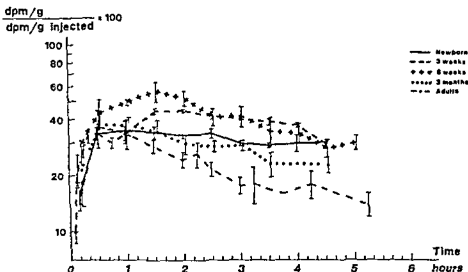


Fig 1 Plasma concentrations of radioactivity at different times after subcutaneous injection of 0 5 mg/kg of atropine Mean from 3 animals ± S E M (adult = 7 animals)

tion there is an increase in the plasma concentrations of radioactivity from the first blood sample at 10 minutes to 30 minutes after injection in the two oldest groups, while the concentration curves in the 3 and 6 weeks old dogs

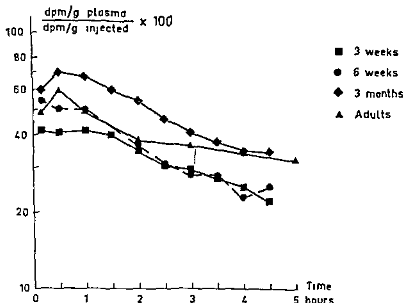


Fig 2 Plasma concentration of radioactivity at different times after intravenous injection of 0 5 mg/kg of atropine In 3 months and adult animals mean from 2 experiments

Table 1

Half lives of plasma radioactivity after 0 5 mg/kg of atropine

Age group	Administration	Number of animals	$T_{1/2}$* min	Variation**
Newborn (A)	s c.	3	690	500-1120
3 weeks (B)	s c	3	320	270- 390
	i v	1	310	285- 340
6 weeks (C)	s c.	3	210	180- 250
	i v	1	210	170- 430
3 months (D)	s c	3	290	220- 400
	i v	2	215	200- 230
Adult (E)	s c ***	7	130	100- 210
			730	600- 920
	i v	2	150	100- 280
			750	440-2500

* Values from 60 min and onwards used in groups A, D and E, in groups B and C from 90 min

** Calculated as $\frac{0\ 69315}{\bar{K} \pm \text{S E M}}$

*** Partly from ALBANUS *et al* (1968)

show almost a plateau during the first hour (fig 2) There are no significant differences in the plasma half-life of radioactivity between subcutaneous and intravenous injection (table 1) Furthermore, the differences between the different age groups are small, except for the newborn dogs which show a very slow elimination of radioactivity from 1 to 5 hrs after subcutaneous injection (half-life 11 5 hrs) However, elimination of the unchanged drug is faster, since chromatography revealed that all the radioactivity in the plasma represented unchanged atropine at 0 5 hr, while the corresponding values for 4 and 8 hrs were 60 and 30 to 40 per cent, respectively (table 5) No difference was found in the plasma concentrations between the newborn dogs used for the distribution studies and the plasma concentration studies Since the former group did not receive pentobarbital before the experiments, it is not likely that this drug interferes with the elimination of radioactivity from the plasma

Binding to plasma protein

In vitro incubation of heparinized plasma from adult animals with atropine in concentrations from 0 01 to 100 μg/ml plasma showed a binding to plasma proteins of between 16–20 per cent, as determined by ultrafiltration, without concentration dependence of the binding (table 2) The plasma binding of

radioactivity in dogs injected with labelled atropine was between 12 and 17 per cent and did not differ significantly between the age groups (table 2) The binding obtained after *in vitro* incubation with atropine was slightly larger than that found for radioactivity *in vivo* This might depend on the presence of metabolites of atropine in plasma, which are not bound to plasma proteins to the same extent

Urinary excretion

The urinary excretion of radioactivity was 15 per cent at 2 hrs and 25 per cent at 4 hrs in the newborn and six weeks old animals, i e about half of that in the adults (fig 3) The administration of atropine did not appear to affect the urinary pH, which varied between 5 8 and 7 4 Furthermore, an adequate diuresis was maintained throughout the experiments

The radioactive metabolites were routinely separated by paper chromatography in butanol acetic acid (5 1) saturated with water Thin layer chromatography and high voltage electrophoresis in other solvent systems (Conc ammonia Methylacetate Propanolol (3 45 35) on DC-Fertig platten, Silica gel F_{254} (Merck, Darmstadt, Germany) Acetic acid (2N) with formic acid (0 6 N) final pH 2, or borate buffer (0 5 M), final pH 10, on Whatman No 1, 20 V/cm, 2 hrs) reported by others to separate atropine metabolites (WERNER & SCHMIDT 1958, ALBANUS *et al* 1969) gave a less efficient separation

During the first hour, no metabolites were detected in the urine samples

Table 2

Plasma binding of atropine

*In vivo**	Age group	Per cent binding mean ± S E M.	Number of animals
	Newborn	17 ± 6	4
	6 weeks	12 ± 2	5
	3 months	17 ± 3	2
	Adult	13 ± 4	6
In vitro	Concentration of atropine µg/g plasma		Number of experiments
	0 01	19 3 ± 1 6	6
	0 1	18 5 ± 1 5	5
	1 0	16 1 ± 0 3	5
	10	17 1 ± 1 6	2
	100	19 4 ± 1 2	3

* Radioactivity in plasma 0 6–4 hrs after injection

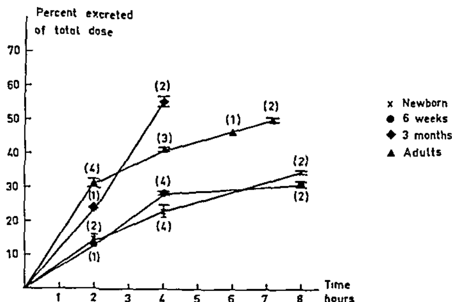


Fig 3 Urinary excretion of radioactivity in relation to injected amount at different intervals after subcutaneous injection of atropine *Mean ± S E M (n) = number* of animals

Table 3

Paper chromatographic evaluation of urinary metabolites after subcutaneous injection of 0 5 mg/kg of atropine (per cent of recovered)

Age group	Litter	Collection period	Rf values					
			I 0 03– 0 07	II 0 20– 0 34	III 0 49– 0 58	IV 0 63– 0 69	V (atropine) 0 60– 0 77	VI 0 79– 0 89
Newborn	1,2	0–60					100,100	
	3						100	
	1	120–240		27,21			73,62	–, 17
	1			19,21			65,65	16,14
	3,4	240–480	20,34	15,35			65,31	
6 weeks	5	0–30					100,100	
	5	0–60	<5	5		10	85	
	2	120–240		22,47			78 53	
	5	240–480	5	20	5		70	
Adult		0–30		2, –	–, 6	– 5	98,87	–, 3
				7, 5	17		83	
		120–240			1, 3		93, 93	
		240–480		23	3	12	61	

Table 4

Organ radioactivity concentrations* after subcutaneous injection of atropine

Organ	Time after injection (min)	Age Group Newborn	6 weeks	Adult
Liver	30	132 180 85	456, 687	319, 241**, 232**
	480	142, 177	275	236
Kidney	30	212,254 91	508, 696	177
	480	45, 62	45	46
Submandibular	30	114,125,55	177, 116	231
Gland	480	31, 27	42, 52	19
Skeletal	30	28, 38 15	57, 87	59
Muscle	480	10 12	4 2 5 6	6 0
Heart	30	51 49,33	140 100	63 100**, 62**
(apex)	480	11, 14	8 8, 8 6	12
Heart	30	53,56,35	103, 103	56
(auricle)	480	13, 14	8 6, 7 7	17
Bile	30	12, 76	55, 230	709, 460**
	480	269, 280	1730	5770, 14300**, 15500**

* Expressed as $\frac{\text{d p m /g tissue wet weight}}{\text{d p m./g injected}} \times 100$

** From ALBANUS *et al* (1968)

Mean of underlined values significantly ($P < 0\,1$) higher or lower than corresponding mean in the newborn

from the newborn animals, while in the 6 weeks old and adult animals 2–17 per cent of excreted radioactivity was in the form of metabolites (table 3) During the 2–4 hour collection period, about 35 per cent of the urinary radioactivity was recovered as metabolites in the newborn and 6 weeks old dogs, while the corresponding figure in the adult was about 10 per cent The main urinary metabolite had an Rf of 0 20–0 34 in all three age groups During the 4–8 hour collection period 30–70 per cent of the excreted radioactivity appeared in the form of metabolites and no significant differences were found between the age groups

Distribution of radioactivity

The concentration of radioactivity in the liver, kidney, submandibular gland, heart, skeletal muscle, bile and brain was investigated at 0 5, 1½ and 8 hrs in the puppies and at 0 5, 2 and 8 hrs in the adult

subcutaneous injection of 0 5 mg/kg of atropine With regard to the maximal concentration of radioactivity, the organs can be divided into three groups, one group, with a maximum concentration 3–10 times that of plasma, consisting of liver, kidney and submandibular gland, one group with a maximum concentration close to the plasma level, consisting of skeletal muscle and the heart tissues (table 4), and a third group, i e the brain, which has a maximum concentration of about one half of that of plasma (table 6) There were no significant differences in organ concentrations of radioactivity between the different age groups except at 0 5 hr after the injection, when the concentrations in the peripheral organs were generally lower in the two youngest age groups (table 4) In the liver, kidney and salivary gland, maximal concentrations were usually found at one or two hours after the injection, while in the heart and skeletal muscle the highest concentrations were found at the shortest period investigated, i e 0 5 hr, except for the newborn which showed the highest concentration at 1 hr Fig 4 shows the time course for the concentration of radioactivity in two typical organs, i e liver and heart muscle, for the different age groups The concentration in the bile was higher than in any tissue The concentrations increased with time and were 3 to 10 times higher in the adult animals than in the newborn at all times investigated (table 4) A single metabolite (Rf=0 03–0 07) was responsible for 70 and 90 per cent of the radioactivity in the bile, collected at four hours after the injection in a three and in a six weeks old dog (table 5)

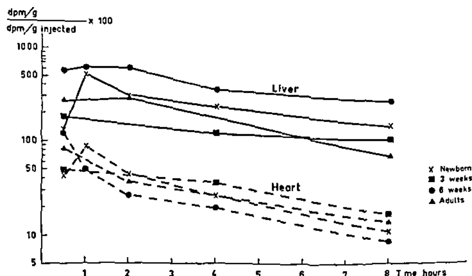


Fig 4 Organ concentrations of radioactivity at different times after subcutaneous injection of atropine Mean from 1–4 animals

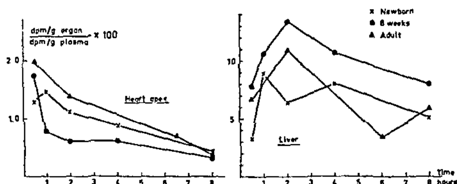


Fig 5 Organ concentrations of radioactivity in relation to plasma concentration after subcutaneous injection of atropine Mean of 1–4 individual ratios

The ratio between organ and plasma radioactivity gives an estimate of the rate of penetration of radioactivity into the tissues These ratios showed two different types of course, in the liver and submandibular gland there was an increase during the first 1 to 2 hrs while in the other peripheral organs the highest ratio was found at the shortest interval, i e 0 5 hr, except for the

Table 5

Paper chromatographic evaluation of atropine metabolites in some organs

Organ	Age group	Litter	Time hrs	Per cent of recovered activity I	II	III	(atropine)[1]
Plasma*		3,4	0.5				*100 100*
	Newborn	4	4	30,15	20, –	– 50	*50, 35*
		3		20	20	–	*60*
		4,3	8	40,25	20,25	– 20	*40 30*
	Adult		4	30 47			*70, 53*
Muscle*	Adult		8	15			*85*
Brain*	Newborn		8				*100*
	Adult		8				*100*
Bile	3 weeks	4	4	70			*30*
	6 weeks	4	4	90			*10*

[1] Italized values indicate strips corresponding to atropine strips in control chromatograms

* Investigated by combustion of 3 cm paper strips Length of chromatograms 27–30 cm

Table 6

Brain concentrations* of radioactivity 2 hrs after injection of atropine

	Newborn		6 weeks		Adult	
Choroid plexus	35 ± 3	(38)	54 ± 1	(61)		
Hemisphere**						
slice I	20 ± 3	(24)	21 ± 2	(27)	19	(32)
slice II	23 ± 4	(26)	29 ± 1	(36)	19	(29)
slice III	24 ± 4	(27)	31 ± 1	(38)	23	(35)
slice IV (cortex)	26 ± 2	(30)	34 ± 2	(41)	25	(36)
Midbrain	31 ± 4	(36)	—		27 ± 1	(45)
Cerebellar (cortex)	27 ± 6	(31)	—		27 ± 3	(38)

Values within brackets give d p m /ml tissue water instead of wet weight

* Expressed as $\frac{\text{d.p m /g tissue wet weight}}{\text{d p m./g injected}} \times 100$

** Slice I closest to the ventricle

Mean of values from 1–2 dogs (adult) and 2–3 dogs (other two groups) ± S E M

newborn puppies where the highest ratio was found at 1 or 2 hrs Fig 5 gives the time course of the ratio for liver and heart muscle There were no significant differences between the curves for the different age groups for the organs In adult dogs, the radioactivity in skeletal muscle was, to 85 per cent, in the form of the unchanged drug 8 hrs after injection, while the corresponding figures for plasma were only 53–70 per cent as early as at 4 hrs (table 5)

There were no significant differences between the age groups in the concentration of radioactivity in the brain (hemispheres, midbrain and cerebellar cortex, table 6) Moreover, all of the radioactivity in the brain was recovered as unchanged atropine both in newborn and adult dogs (table 5) In all age

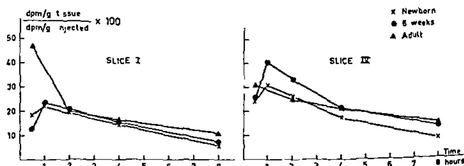


Fig 6 Brain hemisphere concentrations of radioactivity after subcutaneous injection of atropine Slice I close to the lateral ventricle slice IV cerebral cortex Mean from 1–3 animals

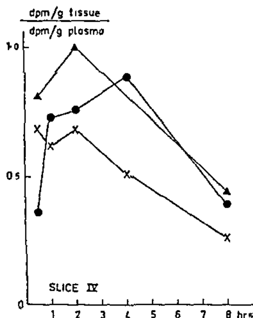


Fig 7 Concentration of radioactivity in cerebral cortex in relation to plasma concentration at different times after subcutaneous injection of atropine Mean of 1–3 individual ratios

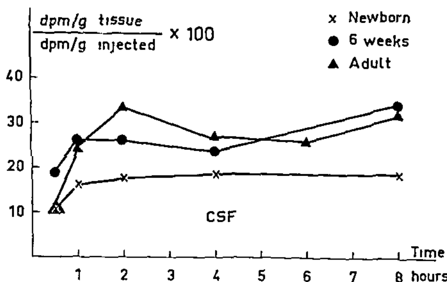


Fig 8 Concentration of radioactivity in cerebrospinal fluid at different intervals after subcutaneous injection of atropine Mean from 1–3 animals

groups, a concentration gradient falling from the cerebral cortex to the lateral ventricle was evident at all the times investigated Maximum brain concentrations were found after one or two hours in the newborn and six weeks old animals In the adult dogs the concentrations found at 0 5 to 2 hrs were rather similar, except for the tissue slice closest to the lateral ventricle, which showed a significantly higher content at 0 5 hr (fig 6) In the newborn and 6 weeks old dogs, the concentration in the slice closest to the ventricles at this time was also slightly higher than in the adjacent slice The ratios between the brain tissue and plasma radioactivity generally showed a peak between one and four hours and were below one (fig 7) In the newborn puppies these ratios showed a more flaccid time course The radioactivity in CSF showed a time course different from that in the brain in that no definite maximum appeared before 8 hrs (fig 8) At this time, the ratio between CSF and plasma radioactivity was close to one in the six weeks old and adult dogs, while it was 0 6 in the newborn animals

Distribution in relation to relative tissue water content

It is a well known fact that the relative water content decreases with age This was also found in the different organs in the present investigation

Table 7

Tissue content of water (per cent of wet weight)

Tissue	Age Group				
	Newborn	3 weeks	6 weeks	3 months	Adult
Liver	74	75	72	67	66
Kidney	84	82	81	74	81
Submandibular gland	80	-	77	75	74
Skeletal muscle	80	72	76	68	69
Heart apex	79	82	79	77	73
Heart auricle	81	83	82	78	73
Choroid plexus	91	86	89	88	81
Brain slice I	85	88	79	74	60
II	90	88	81	78	65
III	89	86	82	79	66
IV	88	86	82	80	69
Midbrain	87	85	76	71	60
Cerebellar cortex	87	85	83	81	72
Number of animals	13-17	2-3	7	2	4

S E M less than 3 for all samples

(table 7) However, in the peripheral organs the changes are rather small, 3–11 per cent Thus, expressing the organ concentrations in these organs per ml of tissue water does not essentially alter the distribution picture In the brain, on the other hand, the changes are larger, 15–28 per cent, and expressing brain concentrations per ml water decreases the concentration of radioactivity in the hemisphere in the two youngest age groups in relation to the adult (table 6, values within brackets) Thus the concentration gradient demonstrated within the hemispheres is still evident The changes with time in this gradient, when expressed as the concentration ratio between the most superficial slice and that closest to the ventricle, is shown in fig 9 The gradients are most pronounced at 0 5 and 8 hrs after injection in the newborn and 6 weeks old dogs In the adult dogs a very low ratio is found at 0 5 hr due to the high peri-ventricular concentration at this time The concentration of radioactivity in the parts close to the lateral ventricle is higher than the CSF concentration until 2 hrs after the injection Thereafter, the CSF concentration seems to be higher, but no search for metabolites in the CSF was carried out because of technical difficulties

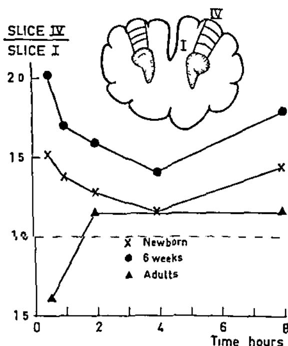


Fig 9 Ratios between concentrations of radioactivity per g tissue water in cerebral cortex and periventricular tissue at different times after subcutaneous injection of atropine Mean of 1–3 individual ratios

Discussion

There appears to be no simple relation between the plasma half-life of radioactivity and the rate of urinary excretion of radioactivity, since only the newborn group shows a longer plasma half-life, while even the six weeks old dogs excrete radioactivity more slowly than the adult The finding of a later maximal plasma concentration and generally lower organ concentration in the two youngest age groups than in the older groups at 0 5 hr after subcutaneous injection, suggests a slower absorbtion of the drug from the injection site in these two groups A larger injection volume was used in the youngest two groups than in the older (1 0 ml/kg as against 0 1 ml/kg) However, the opposite effect on absorption rate would be expected, since it has been shown that increasing the atropine concentration decreases the clearance from the injection site (SUND & SCHOU 1964) Anyhow, since there were no significant differences in plasma or organ concentrations of radioactivity between the newborn and adult animals at one hour, it is not likely that the longer half-life in plasma radioactivity in the newborn dog between 1 and 5 hrs is due to a continuous absorption of atropine from the injection site

The low concentration of radioactivity in the plasma found at the shortest investigated time, 10 min, after the intravenous injection, reveals that atropine is very rapidly cleared out of the plasma during the first ten minutes and there seems to be no difference in that respect between the age groups investigated Such a rapid clearance has also been found in mice (EVERTSBUSCH & GEILING 1956, WERNER & SCHMIDT 1968) This must be due to an accumulation in certain organs which contain concentrations well above that of the plasma, i e the liver, kidney and submandibular gland During the first one to two hours there is a further uptake of radioactivity in these organs but there were no significant differences between the age groups regarding the concentrations of radioactivity in this group of organs from 1 hr and onwards From 10 to 30 min after the intravenous injection there is a plateau or, in the two oldest groups, even an increase in plasma radioactivity The most probable explanation for this is a release of metabolites into the plasma which is more pronounced in the two oldest groups However, at four hours after the subcutaneous injection there were no significant differences between newborn and adult dogs in the fraction of metabolites in the plasma These findings indicate that the longer plasma half-life of radioactivity in the newborn dogs is not due to a slower uptake in or faster release of radioactivity from the tissues In addition to liver, kidney and salivary glands studied in this investigation, high concentrations were found in the lungs, small intestine and spleen, and also in the gastrointestinal contents of mice (EVERTSBUSCH & GEILING 1956, WERNER & SCHMIDT 1968,

GOSSELIN *et al* 1955, ALBANUS *et al* 1968a, ALBANUS *et al* 1969) The concentration of radioactivity in these tissues has not been determined in the present investigation and it cannot be excluded that changes with age in the accumulation of atropine in these tissues are partly responsible for the longer half-life in the newborn dogs Bile concentrations were higher in adult and six weeks old dogs than in the newborn However, the three weeks old puppies had bile concentrations similar to the newborn in spite of a shorter plasma half life of radioactivity The amount of metabolites in the urine in the different age groups does not indicate that quantitative differences in the rate of biotransformation of atropine are responsible for the longer plasma half-life of radioactivity in the newborn puppies

A slower urinary excretion of atropine was found in the newborn and six weeks old dogs Evidently this does not depend on an effect of atropine on urinary pH since these changes were small and the pH never exceeded 8, which seems to be a critical border line for the excretion in the dog (ALBANUS *et al* 1968b) As discussed above, the differences found in the relative rate of metabolic degradation are small and cannot explain the age difference in excretion rate To our knowledge, there are no conclusive data on the effect of age on glomerular filtration rate for the dog However, the data for inulin clearance for 2–8 weeks old puppies reported by HOOK *et al* (1970) are in good agreement with clearence data for adult dogs when related to the body weight In man and sheep, there is a rather wide scatter but no certain difference in filtration rates in relation to body weight between newborn and adults (RUBIN *et al* 1949, ALEXANDER & NIXON 1962) It has been shown that part of the atropine excretion is due to an active mechanism (ALBANUS *et al* 1968a) and it is also known that renal transport mechanisms for *p-amino hippurate* are of an immature nature during the first eight weeks of life in the dog (HOOK *et al* 1970) It thus seems possible that age differences in active excretion are responsible for the differences in excretion rates

There was a continuous decrease from 0 5 to 8 hrs in the ratio between tissue and plasma radioactivity in all the organs except the liver and submandibular gland This might be due to the fact that an increasing fraction of the plasma radioactivity is in the form of metabolites with a limited penetration into the tissues However, the change in ratio is generally larger (3–7 times in 8 hrs) than would be anticipated from the plasma content of metabolites (60–70 per cent at 8 hrs, giving a maximal change in ratio of 3 3) It cannot be excluded that the chromatographic procedure does not separate all metabolites from atropine

The penetration of atropine into the brain is slow, since the highest brain concentrations were found at one or two hours after the injection There is no indication that atropine penetrates more readily into the brains of newborn puppies, on the contrary brain plasma ratios are usually higher for adult

animals than for puppies at 0 5 hr after the injection, when all of the plasma radioactivity represents unchanged atropine in the newborn dogs An exception to this slow penetration was the tissue closest to the lateral ventricles in the adult animals, where a high concentration in comparison with other parts of the brain was found after 0 5 hr In newborn and 6 weeks old animals too the concentration in this tissue was somewhat higher than in the underlying tissue A similar distribution has also been reported in an autoradiographic study in mice (ALBANUS *et al* 1968b) The short duration of this high concentration suggests a circulatory origin However, the water content of the slice closest to the ventricles is comparably low, indicating a low fraction of grey matter, and the blood flow is about five times greater in grey than in white matter (ROTH *et al* 1970) Because of the higher concentration of radioactivity in the brain close to the ventricles than in the CSF during the first two hours, atropine cannot pass into the brain by this route by simple diffusion An active uptake of atropine has been shown in mouse brain slices (SCHUBERTH *et al* 1966) However, both the time course of the concentration in the peri-ventricular tissue in relation to CSF, and the existence of a concentration gradient in the hemispheres falling towards the ventricles, are at variance with the assumption that an active uptake mechanism is of importance for the high peri-ventricular concentrations of radioactivity at 0 5 hr

The CSF concentrations were lower in newborn animals than in adults especially in relation to plasma As reported previously for adult animals (ALBANUS *et al* 1968a), the CSF concentration of radioactivity in all age groups reaches a plateau which remains rather stable during the following 8 hrs The mechanism responsible for the plateau is not evident A possible explanation for the CSF plateau is a saturated secretion of atropine into the CSF (ALBANUS *et al* 1968a) The lower values in the newborn might then reflect a lower transport capacity, in accordance with the immaturity of the renal transport systems WANG & TAKEMORI (1971) reported that, in rabbits, morphine, another tertiary amine, is secreted into the CSF by a carrier mediated transport, while atropine is cleared out of CSF by such a mechanism in the same species (AQUILONIUS & WINBLADH 1972) In dogs, no evidence for a carrier mediated elimination of atropine out of the CSF has been found in ventriculocisternal perfusions (AQUILONIUS & WINBLADH, unpublished observations) but the transport into the CSF has not been investigated in this respect Another explanation for the CSF plateau is that the atropine in the CSF is mainly diffused from the brain substance CSF acts as a "diffusion sink" for some extra cellular substances (POLLAY & KAPLAN 1970) and preliminary data on hemisphere concentration gradients of atropine in dogs (ALBANUS & WINBLADH 1969) seem to support the assumption that this also applies to atropine The finding of higher concentrations of the drug in the

CSF than in the brain after 2 hrs need not necessarily contradict such a mechanism, since the concentration in the brain compartment, from which this diffusion could take place, might very well be higher than the CSF concentration However, the establishment of the hemisphere gradient as early as at 0 5 hr and the high peri ventricular concentrations indicate that other factors are of importance for the establishment of these gradients

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REFERENCES

Albanus L. A Sundwall B Vangbo & B Winbladh The fate of atropine in the dog *Acta pharmacol et toxicol* 1968a, 26 571–58?

Albanus L. L Hammarstrom A Sundwall S Ullberg & B Vangbo Distribution and metabol sm of H^3-atropine in mice *Acta Phys ol Scand* 1968b 73 447–456

Albanus L., A Sundwall & B Vangbo On the metabol c disposition of methylatropine in animals and man *Acta pharmacol et toxicol* 1969 17 97 111

Albanus L. & B Winbladh Distribution of atropine and methylatropine in the brain substance *Abstr IV Int Congr Pharmacol* Basel 1969 443–444

Alexander D P & D A Nixon Plasma clearence of p aminohippuric acid by the kidneys of foetal neonatal and adult sheep *Nature* 1962 194 483–484

Aquilonius S M & B Winbladh Cerebrosp nal fluid clearence of choline and some other amines *Acta Physiol Scand* 1972 85 78 90

Borgå O D L Azarnoff G Plym Forshell & F Sjoqvist Plasma protein bind ng of tricyclic antidepressants in man *Biochem Pharmacol* 1969 18 2135 2143

Eriksson K H & B Winbladh Choro d plexus uptake of atropine and methylatropine *in vitro Acta Physiol Scand* 1971 83 300–308

Evertsbusch V & E M K Geiling Studies with radioactive atropine Distribution and excretion patterns in the mouse *Arch int pharmacodyn* 1956 CV 175 192

Gabourel J D & R E Gosselin The mechanism of atropine detoxication in mice and rats *Arch Int Pharmacodyn* 1958 115 416–432

Goldenthal E J Compilation of LD_{50} values in newborn and adult animals *Toxicol appl Pharmacol* 1971 18 185 207

Gossel n R E J D Gabourel S C Kalser & J H Willis The metabolism of C^{14} labelled atropine and tropic acid in mice *J Pharmacol Exp Therap* 1955 115 217–224

Hook J B H E Williamson & G H Hirsch Functional maturation of renal PAH transport in the dog *Can J Physiol Pharmacol* 1970 48 169–175

Kalser S C J H Wills J D Gabourel R E Gosselin & C F Epes Further studies of the excretion of atropine-alpha C^{14} *J Pharmacol Exp Therap* 1957 121 449–456

Kanda Z Über die Beziehung zwischen dem Lebensalter und der Toleranz fur Atropin bei weissen Ratten *Jap J Med Sci Pharmacol* 1941 14 13 17

Obata, T Über die Beziehung der Toleranz der weissen Ratten gegen verschiedene Arzneimittel zu ihrem Lebensalter *Jap J Med Sci Pharm* 1931, 5, 83–84
Ostle, B *Statistics in research* Iowa State College Press, Iowa 1954
Pollay, M & R J Kaplan Effect of the CSF sink on thiocyanate concentration gradient in brain *Amer J Physiol* 1970, 219, 802-808
Roth, J A, A J Greenfield, S Kaihara & H N Wagner Total and regional cerebral blood flow in unanesthetized dogs *Amer J Physiol* 1970, 219, 96–101
Rubin M J, E Bruck & M Rapoport Maturation of renal function in childhood Clearence studies *J Clin Invest* 1949, 28, 1144–1161
Schuberth, J, A Sundwall, B Sorbo & J O Lindell Uptake of choline by mouse brain slices *J Neurochem* 1966, 13, 347–352
Sund, R. B & J Schou Absorption of atropine, anticholinergic agents as inhibitors of absorption from muscles *Acta pharmacol et toxicol* 1964 21, 339–346
Wang J H & A E Takemori The exchange of morphine between blood and CSF in rabbits *Fed Proc* 1971, 30 (2), 701
Werner, G & H L. Schmidt Chemische Analyse des Stoffwechsels von Atropine bei der Maus *Hoppe Seyler Z Physiol Chem* 1968, 349, 677–691
Willberg M A Die natürliche Resistenz einiger Tiere dem Atropin gegenuber *Bio chem Z* 1914 66, 389–407

Acta pharmacol et toxicol 1973, 32, 65–82

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Postnatal Development of Central Effects of Atropine and Oxotremorine in Dogs in Relation to Brain Development

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Abstract The central effects of atropine, methylatropine and oxotremorine have been investigated in dogs at five different ages, i e newborn 3 weeks, 6 weeks, 3 months and adult The evaluation of the central effects has been made by behavioural and neurological tests especially designed for the different age groups In adult dogs 0 5 mg/kg of atropine and 20 mg/kg of methylatropine cause typical behavioural disturbances i e ataxia non responsiveness non retreating behaviour and increased motor activity, while oxotremorine 0 025 mg/kg produces a pronounced tremor In the newborn dogs no central effects can be detected with atropine, 0.5–5 0 mg/kg or methylatropine, 20 mg/kg Furthermore tremor can not be elicited with 0 01–1 0 mg/kg of oxotremorine in this age group Among the 3 weeks old puppies the neurologically and behaviourally most mature animals show central effects similar to those in the adult following 0 5 mg/kg of atropine, while more immature dogs show no symptoms even after 5 0 mg/kg The sensitivity to the tremorogenic affect of oxotremorine is the same in this age group as in the adult dogs At six weeks and three months of age the effects of the two drugs are the same as in the adult animals In order to evaluate the development of the central cholinergic system the activities of choline acetyltransferase and cholinesterases have been determined in whole brain, cerebellum caudate nucleus and cerebral cortex in the different age groups An increase is found in relative enzyme activity from birth up to adulthood

The changes in the effects of atropine and oxotremorine are discussed in relation to the development of the two enzymes, and to other biochemical, pharmacological electrophysiological and behavioural data known for the dog It is suggested that the change in sensitivity to the two drugs reflects the development of the central cholinergic system which parallels the general development of the brain

Key words Atropine – behaviour, animal – acetylcholinesterase – choline acetyl transferase – age factors

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There are few experimental data on changes with age in the effects of anticholinergic drugs The only effect which has been more

vestigated is that of atropine on heart rate In the species investigated, which included dogs, sensitivity to the cardiac acceleratory effect of atropine increased with age (LHOTAK 1911, SCHLOSSMAN 1937, FOX 1966a, ADOLPH 1967 & 1971, ROZANOVA 1968) The few available data on age differences regarding the central effects of anticholinergic drugs are rather diverse, but nevertheless favour increased effects with age ALBERTONI (1882) reported that young dogs (30–50 days) showed less pronounced behavioural effects with atropine than adult dogs Atropine facilitated imprinting in 8 hrs-old chicks, but hampered it at 14 hrs and later (KOVACH 1964) In rats, scopolamine was without effect on locomotor activity until 20 days of age (CAMPBELL *et al* 1969) and did not enhance amphetamine gnawing in 10-day olds, but did so in adult animals (McGEER & McGEER 1971) Further, local application of atropine on the primary sensory cortex in newborn kittens did not influence cortical primary responses at all or less than in adult animals (BAKLAVADZJAN & ADAMJAN 1964)

Anticholinergic drugs produce a typical behavioural syndrome in adult dogs, called the central anticholinergic syndrome (CAS, ALBANUS *et al* 1969), which is suitable for the study of central cholinolytic effects This syndrome consists of muscular incoordination, here called ataxia, non responsiveness, non-retreating behaviour and increased locomotor activity Furthermore, there are no differences in the brain concentrations of atropine between puppies and adult dogs (WINBLADH 1972) These facts seemed to make dogs suitable for a study of changes in the central effects of atropine during development

From studies on isolated guinea pig and human ileum and the human ductus arteriosus, it has been concluded that cholinergic receptor characteristics do not change from the foetal to the adult stage (BOREUS & McMURPHY 1971, McMURPHY & BOREUS 1971), but nothing is known about the central receptor sites in this respect However, in most of the above mentioned studies it is claimed that the lower sensitivity to anticholinergic drugs in young animals depends on an immature cholinergic system We thus found it relevant to relate changes in the central effects of atropine to the effect of oxotremorine, a centrally active muscarinic agonist (see reciew by KARLÉN 1970) and to the relative activities in some parts of the brain of acetylcholinesterase (AChE, EC 3 1 1 7) and choline acetyltransferase (CAT, EC 3 1 1 8), two important enzymes in acetylcholine (ACh) metabolism

Methods

Behavioural tests

Beagle puppies of both sexes were used at the following ages newborn (1–5 days) 3 weeks (18 25 days) 6 weeks (40–50 days) 3 months (83–100 days) and adult (over 300 days) During the behavioural tests the newborn animals were kept in boxes of

Table 1.

Registration schedule for newborn and 3 weeks old puppies

Peripheral *Rectal temperature, *heart rate *salivation dry, decreased, normal, hypersalivation

Rating	2	1	0
*Placing reaction	absent	uncertain	immediate
forelegs			
hindlegs[1]			
*Rooting[2]	absent	slow	pronounced
*Righting	absent	slow	immediate
*Negative geotaxis[3]	absent	uncertain	immediate
*Sucking[4]/food response	absent	slow	hungry
*Responsiveness[5]	absent	decreased/sleepy	alert
Motor activity			
Sounds		yelping	quiet
Comments[6]	clearly affected	probably affected	normal

* Determined at examinations (see text)
1 Only determined in 3 weeks old puppies
2 Head turning towards stimulated side when gently rubbing cheak
3 Turning with head upwards, when placed on leaning board with head downwards
4 Sucking investigator's finger when put in mouth
5 Change in behaviour on handling In three weeks as well on visual and auditory stimulation
6 Investigator's impression of behaviour

0.3×0 5 m with an external temperature of 24–27° The older animals were allowed to move freely in a special chamber (2 4×4 8 m, 22°) in which they could be observed through a one-way screen At least two animals were tested together in the box or the chamber, one injected with methylatropine 0 5–1 0 mg/kg serving as control Behaviour in animals older than 3 weeks was assessed according to a schedule as described elsewhere (ALBANUS *et al* 1969), while for the youngest two age groups special rating schedules were developed (table 1) During the first hour after injection the examiner went in to the animals (or picked up the newborn animals from the box) and determined some of the parameters (marked with a star in table 1) During the following three hours the animals were examined once every half hour Inbetween the examinations, the animals were watched through the one-way screen for registration of the other parameters

The reflex tests described in table 1 were performed as described by Fox (1964) (see also table 1) In the two youngest groups, the ability to suck the investigator's finger and the attitude towards feeding with a nipple bottle containing milk was also investigated The heart rate was determined by auscultation and salivation by inspection and palpation of the mucous membranes in the mouth The rectal temperature was measured with a thermocouple thermometer Because of the difficulty of evaluating locomotor activity in the two youngest groups, separate experiments on this parameter were performed with an inductive coil meter (Animex®, Farad electronics, Stockholm,

Sweden) In these experiments too a control animal injected with methylatropine was run in parallel with the test animal Activity was registered every fifth minute and the difference between the test and the control animal during at least 3 consecutive one-hour periods was evaluated by means of a paired t test (OSTLE 1954)

The central effects of oxotremorine were determined in a similar fashion as for atropine in the first series of experiments but the two parameters tremor and stiffness of the extremities were added to the scheme In the later experiments the scheme was simplified for the experiments involving the three oldest groups (see results table 7) The effects on locomotor activity in the two youngest groups were investigated with the Animex apparatus as described above To avoid any peripheral effects of oxotremorine, 0 5 mg of methylatropine was given 0.5 hr before the injection of oxotremorine

All the experiments were performed as blind trials using methylatropine as the control substance in the atropine experiments, and 0 9 % saline in the oxotremorine experiments The behavioural tests were performed by one investigator In one series of experiments an investigator with considerable experience from identical studies in adult dogs also participated in evaluating the behaviour of the dogs There were no significant differences in the test protocols between the different investigators In another series of experiments the comment section (table 1) was omitted and the author made an evaluation of the behavioural disturbance from the remainder of the test protocol without other knowledge of the actual experiment These statements were then compared with separate statements on behavioural disturbances by the usual investigator A good correlation was found between the statements

All drugs were dissolved in distilled water and given subcutaneously in the nape of the neck Injection volumes were 1 0 ml/kg in the two youngest groups and 0 1 ml/kg in the remainder of the dogs Doses were calculated as atropine sulphate and methyl atropine nitrate

Enzyme determinations

Dogs were killed with pentobarbital (mebumalum NFN) and the brains rapidly removed after section of the medulla at the level of the foramen magnum and chilled to 6–8° in a refrigerator The following parts were taken for enzyme analysis whole brain without cerebellum cerebellum parietal cortex from the gyrus lateralis (NAP) and the caudate nucleus In the two youngest groups dissection of the cortex, and particularly of the caudate nucleus was difficult because of the poor demarcation between grey and white matter The brain samples were homogenized in 3 volumes of water in a Potter Elvehjem homogenizer and stored at −20° until analysis

The assay of CAT activity was performed as described by MANNERVIK & SORBO (1970) with the following three modifications 1 The concentration of eserine was increased five times to ensure that no enzymatic hydrolysis of formed acetylcholine could occur This did not influence the CAT activity 2 The butanol concentration was increased ten times since this concentration was found to give optimal CAT values Furthermore the higher butanol concentrations reduced the property of the enzyme preparation to lose activity on dilution 3 The acetylation was stopped by the addition of 0 5 ml of a solution of 1 5 per cent tetraphenyl boron (Kalignost Merck Germany) in 3 heptanone (FONNUM 1969) and shaking on a mixer for 30 seconds The organic phase separated spontaneously and 0.3 ml was immediately mixed with a scintillation solvent and assayed by liquid scintillation as described elsewhere (ERIKSSON & WINBLADH 1971) Addition of labelled acetylcholine to the reaction mixture gave a total recovery of 61 ± 1 per cent (mean ± S E M of five determinations) in the organic phase Blank

values were below 20 per cent of the lowest activities measured Standard incubation time was 20 min and the time curve was approximately linear between 10 and 30 min for both newborn and adult animals When more than 15 per cent of added acetylcoenzyme A was consumed during the twenty m nute incubation period the tissue homo genate was further d luted with water in order to obtain zero order kinetics (SCHUBERTH & SÖRBO 1971) Sensitivity to dilution of the enzyme activity was sligthly greater in homogenates from mature brains than from immature brains The maximal error intro duced by the dilutions was below 15 per cent The CAT determinations were carried out in duplicate the average difference between the two determinations being 4 7 ± 0.5 per cent (S E.M of 40 pairs)

The activity of the cholinesterase system (ChE) in the homogenates was investigated with acetylthiocholine as substrate using the colorimetric method of ELLMAN *et al* (1961) Spontaneous hydrolys s was below ten per cent of the enzymatic in all the samples In this analysis too the homogenates with the highest activities had to be diluted The mean hydrolysis rate over 15 min was measured Since this method also measures the so-called unspecific cholinesterases the homogenates from newborn and adult dogs were also assayed by the above method but with butyrylthiocholine (Koch Light Lab Inc Colnbrook England) which is not hydrolysed by AChE, as substrate The tissue content of potass um was analysed by flame photometry after dissolution in nitric acid as described elsewhere (WINBLADH 1972)

Results

Peripheral effects of atropine

Atropine already inhibited salivation in all age groups at the first time investigated, i e 15 min The duration of the symptom was rather similar after the administration of 0 5 mg/kg in all the groups with the exception of the newborn which had a longer duration (table 2) The period of hypo-

Table 2

Per pheral effects of atropine 0 5 mg/kg

	Age group				
	Newborn	3 weeks	6 weeks	3 months	adult
Mean duration of saliva tory inhibition (min)	> 360	-	140	100	180
Mean duration of increase in heart rate (min)	(185)	250	190	170	220
Mean maximal increase in heart rate (per cent of basal)	(19)*	32	41	75	90
Number of animals	9	3	5	5	6

* not s gnificantly different from control (saline)

salivation was often followed by hypersalivation, varying between 0 5 and 2 hrs The heart rate was not, or only very slightly affected in the newborn group, and in the 3 and 6 weeks old dogs too the effect of atropine was smaller than in adults (table 2)

Central effects of atropine

All adult dogs tested showed the CAS after 0 5 mg/kg In the newborn group no differences could be detected in the scheduled behavioural parameters between the control animals and the animals injected with atropine, even at the highest dose, 5 0 mg/kg However, it must be pointed out that newborn puppies show a rather stereotype behaviour, which makes behavioural evaluation more difficult and probably less accurate than in older animals The animals usually remained calmly together in their box, and when separated they crawled around, often whimpering until they found their littermate When hungry they also crawled around, often whimpering In both instances the puppies were calmed by careful handling, patting and feeding At this age the litters were neurologically rather even, but during the first 2–3 days the animals did not show any negative geotaxic orientation or front paw placing reflex A common finding was that newborn puppies changed in their responses even to the reflex testing during the experiments However, this was equally common in both test and control animals Locomotor activity, as determined by the Animex recordings, was not clearly increased after either 0 5 or 5 0 mg/kg (table 3) although in a few of the individual investigation periods a significant increase was seen

Table 3

Changes in locomotor activity* (Animex) after atropine in newborn and 3 weeks old puppies

Dose	Group	Time period (min)				
		60–0	0–60	60–120	120–180	n
Newborn	0.5	−23	58	44	28	4
	5 0	−18	32	−25	–	3
3 weeks	0.5	0	42**	11	40**	7

Injection at time = 0

n = number of experiments.

* Procedure Control animal injected with corresponding dose of methylatropine always run in parallel with test animal Registration of movements for every 5 min period Mean of one hour period = M

Changes calculated as mean of $\frac{M_{test} - M_{control}}{M_{control}}$ 100 in n experiments

** Significantly larger than control period 60 – 0 $P < 0 1$

Table 4

Number of 3 weeks old animals showing central effects of atropine

Dose mg/kg	0 5			2 0			5 0		
Rating*	2	1	0	2	1	0	2	1	0
Placing	3		3			2		1	3
Rooting	3		3			2	1		3
Righting		3	3			2	1		3
Negative geotaxis	*2*	*1*	*3*			*2*		*1*	*3*
Responsiveness	2	1	3		1	1	1		3
Sounds		3	3		2			2	2
Comments**	2	1	3		1	1	1		3

* Calculated as rating injected – rating control animal (see table 1) at maximal effect
** Investigator's opinion on degree of behavioural disturbance

The behaviour in the three weeks old animals was considerably easier to evaluate, since they showed a more differentiated behaviour with higher spontaneous activity, crawling or walking and even playing with each other Moreover, the attitude towards the examinator was active There were clear-cut differences between different individuals and litters regarding neurologic *maturity, motor activity and social* behaviour In this age group, a variation in the behavioural effects of atropine was seen While three out of six animals showed typical disturbances in behaviour after 0 5 mg/kg, others appeared unaffected even by 5 0 mg/kg (table 4) In one experiment with two littermates, only one reacted to a dose of 0 5 mg/kg The investigator's impression was that the reacting animal was motorically more mature and alert than the non reacting littermate All three investigated members of one litter, which was claimed by the investigator to be unusually active, motorically mature and alert, developed behavioural symptoms after 0 5 mg/kg There were no significant differences in average age or body weight between reacting and non reacting puppies Altogether, 4 out of the 12 animals tested showed behavioural effects When elicited, the symptoms caused by atropine were *similar to the CAS in adult animals*, i e yelping, increased motor activity, loss of sucking, *rooting, placing and some impairment of righting reflexes*, non responsiveness and in two instances non retreating behaviour Ataxia is impossible to evaluate at this stage of development Both the mean values of locomotor activity, and in six out of seven experiments also the individual values, as measured with the Animex apparatus, showed a significant increase lasting for one to three hours (table 3) Three puppies from the above-mentioned atropine-sensitive litter were tested in this way and all showed significant increases in locomotor activity

In the 6 weeks and 3 months old dogs, the CAS schedule for adult animals

Table 5

Behavioural effects of atropine in the three oldest age groups

Number responding / number tested

Age group	Dose mg/kg	Ataxia	Non retreating	Unresponsiveness
6 weeks	0 1	0/3	0/3	0/3
	0 2	2/4 (135)	0/4	1/4 (210)
	0 3	6/6 (75)	3/6 (90)	4/6 (110)
	0.5	2/2 (120)	2/2 (75)	2/2 (120)
3 months	0 2	0/2	0/2	0/2
	0 3	3/3 (120)	2/3 (75)	3/3 (120)
	0.5	3/3 (135)	3/3 (110)	3/3 (135)
Adult*	0 1	0/4	0/4	0/4
	0 2	3/4 (95)	0/4	0/4
	0 3	5/5 (200)	3/5 (150)	3/5
	0 5	7/7 (240)	7/7 (160)	7/7 (135)

* Values from ALBANUS (1970)

Values in brackets give mean duration of symptom in min

adequately describes the symptoms after atropine However, because of the high spontaneous activity, an increase in locomotor activity was usually not evident On the other hand, this high activity and the social behaviour (i e

Table 6

Central effects of methylatropine in the three oldest age groups

Number responding / number tested

Age group	Dose mg/kg	Ataxia	Non retreating	Unresponsiveness
6 weeks	5	0/2	0/2	0/2
	10	2/2 (135)	0/2	1/2 (225)
	20	1/1 (>240)	1/1 (120)	1/1 (>240)
3 months	5	0/2	0/2	0/2
	10	0/2	0/2	2/2 (190)
	20	2/2 (190)	2/2 (145)	1/2 (60)
Adult*	2.5	0/4	0/4	0/4
	10	5/8 (165)	3/8 (120)	3/8 (135)
	20	4/4 (295/2, >315/2)	4/4 (265/2, >315/2)	4/4 (160)

* From ALBANUS (1970)

Values in brackets give mean duration of symptom in min

playing) makes disturbances in behaviour more easy to detect than in adult animals A fully developed CAS was seen after 0 5 mg/kg in all animals in both the six weeks and the three months old groups Table 5 summarizes these data in comparison with data for adult animals reported previously from our laboratory (ALBANUS 1970) It is seen that the threshold dose for the CAS appears to be the same in the three oldest age groups Further, there are no significant differences between the age groups with regard to the onset and duration of the different symptoms (table 5) During a fully developed CAS the placing reflex was lost in one case out of six in the six weeks old group and in two cases out of five in the three months old group The righting reflex was also slowed in these cases In adult animals, placing was usually lost after 0 5 mg/kg, while righting was only slowed No effect on the rectal temperature was seen in any age group

In short, no influence of atropine on behaviour was seen in newborn puppies and only in comparatively mature animals in the three weeks old group, while in the three oldest groups the effects of atropine were the same as regards both symptomatology and duration

Central effects of methylatropine

Methylatropine did not give rise to behavioural effects in the two youngest groups in doses up to 20 mg/kg In the six weeks old and in the three months old dogs, behavioural disturbances were obtained after 10 and 20 mg/kg but not after 5 mg/kg The dose of 10 mg/kg was equipotent with a dose of atropine of 0 2–0 3 mg/kg, while 20 mg/kg elicited a fully developed CAS Table 6 summarizes these behavioural disturbances after methylatropine in relation to previously published values for adult dogs (ALBANUS 1970)

Central effects of oxotremorine

In adult dogs, pretreated with methylatropine 0 5 mg/kg 0 1 mg/kg of oxotremorine elicited a vigorous tremor of 7–12 cycles/sec within a few minutes, which was most clearly visible in the hindlegs although all superficial skeletal muscles seemed to be affected Other symptoms were rigidity of the extremities motor hyperactivity, hyperthermia and panting No saliva tion or bradycardia was seen, indicating that the dose of methylatropine given was adequate In spite of the dramatic symptoms the dogs appeared aware of their surroundings and usually responsive, but sometimes scared of the investigator The parameters assessed are depicted in table 7 The picture for the three and six weeks and the three months old dogs was quite similar to that of the adult, except that no hyperthermia was seen in the 3 weeks old puppies Furthermore, there were no significant differences in the duration of the tremor between these groups (table 8) The symptomatol[illegible] in the newborn group was different and deserves comment No

Table 7.

Central effects of oxotremorine

Number responding / number tested

Age group	Dose	Tremor	Stiffness	Hyper activity	Hyper thermia
Newborn	0 025	0/2	0/2	–	0/2
	0 1	0/2	1/2	–	0/3
	1 0	0/3	1/1	–	0/3
3 weeks	0 01	1/3	0/3	–	–
	0 025	2/3	3/3	–	–
	0 2	6/6	6/6	–	0/2
6 weeks	0 01	0/2	0/2	0/2	0/2
	0 025	1/3	3/3	0/3	–
	0 1	2/2	2/2	2/2	2/2
3 months	0 01	0/2	0/2	0/2	0/2
	0 025	3/3	1/3	0/3	–
	0 1	2/2	2/2	2/2	1/1
Adult	0 01	1/2	1/2	0/2	0/2
	0 025	1/1	0/1	0/2	–
	0 2	3/3	3/3	3/3	3/3

– = not investigated or evaluated

thermia was seen in this group, but with doses over 0 2–0 5 mg/kg the rooting reflex was difficult or impossible to elicit and respiration became jerky and accompanied by a faint whining Geotaxic orientation was inhibited with doses of 0 1 mg/kg or higher Stiffness was difficult to evaluate in this age group because of natural muscular weakness but seemed to be present with doses over 0 1 mg/kg, as was a peculiar scratching movement in the hind-legs Activity (Animex) was increased by 1 0 mg/kg in the newborn group, and by 0 01 mg/kg and larger doses in the three weeks old group (table 9) The increase in activity could at least be partly attributed to the scratching movement in the newborn and to tremor in the three weeks old group

Table 8

Onset and duration of tremor caused by 0 2 mg/kg of oxotremorine Mean ± S E M

Age group	Onset (min)	Duration (min)	Number tested
3 weeks	11 ± 3	140 ± 35	3
6 weeks	2	103	1
3 months	3	150	1
Adult	5 ± 1 5	145 ± 17	3

Table 9

Activity* (Animex) after oxotremorine in newborn and 3 weeks old puppies

Age group	Dose	Time period			
		30–0	0–30	30–60	60–90
Newborn	0 1	−66	13	−63	
	1 0	17	246	111	150**
3 weeks	0 01	−8	41	30	
	0 025	14	162	110**	40

Injection at time = 0

* Calculated as in table 3 n = 3

** Significantly greater than control period P < 0 1

To summarize, no tremorogenic effects of oxotremorine were seen in the newborn dogs but other effects of the drug were observed, while symptomatology and duration of tremor was the same in the older groups

Changes with age in brain CAT and ChE activities

There was an increase in enzyme activity per gram wet weight from birth to adulthood for both enzymes in the different brain portions studied (fig 1) The largest relative increase by far was found in the caudate nucleus Both enzymes change in a similar fashion with age, except in the cerebellum which shows a fall in CAT at three weeks Furthermore, the relative ChE activity in the cerebellum is highest or the second highest of the four brain samples while relative CAT activity is lowest in the cerebellum and cortex The values obtained for CAT from the dogs in each group are rather similar, while the variation in ChE is considerably larger In the newborn puppies, a considerably larger fraction of the ChE activity in the cerebellum and caudate nucleus is due to non-specific cholinesterase, than in the adult dogs

The total enzyme content in the different brain portions show a 15- to 40-fold increase with age (fig 2) Relative water content of the different samples decreases with age, while the relative intracellular volume, as estimated by the potassium content, increases with age (table 10) When the enzyme activities are expressed per mg dry weight, no increase with age is seen except in the caudate nucleus It has been shown that the intracellular potassium concentration in the cat brain does not change with age (YANNET & DARROW 1938) If this is also true for the dog, the potassium content of the different pieces should roughly reflect the intracellular volume When the enzyme activities are expressed as per mg potassium this reduces the age

Fig 1 Changes with age in activity of choline acetyltransferase (CAT) and cholin esterase (ChE) in different parts of the dog brain CAT ordinate gives rate of acetyl choline (ACh) formation and ChE ordinate gives rate of acetylthiocholine (ATCh) hydrolysis Open circles give hydrolysis rate of butyrylthiocholine Each point rep resents the mean from 1–3 animals ± S E M A = newborn, B = 3 weeks, C = 6 weeks, E = adult

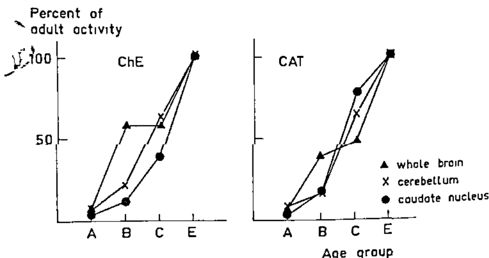


Fig 2 Changes with age in total activity of cholinesterase (ChE) and choline acetyltransferase (CAT) in each brain part expressed as fraction of adult value A = newborn, B = 3 weeks, C = 6 weeks, E = adult

Table 10

Dry weight and potassium content of brain samples

Age group		Newborn	3 weeks	6 weeks	Adult	$\frac{\text{Adult} - \text{newborn}}{\text{newborn}}$ 100
Whole brain	DW[1]	12	13.5	14 5	25 5	113
	K+[2]	4 2	4 0	3 7	4 7	12
Cerebellum	DW	15 5	17	21	34 5	123
	K+	4 3	4 6	4 4	5 0	16
Cortex	DW	13	16 5	17	28	115
	K+	4 1	4 7	5 1	5 4	32
Caudate	DW	12	17	18.5	21.5	79
Nucleus	K+	3 7	4 4	4 6	4 8	30

1 DW = dry weight expressed as per cent of wet weight
2 K+ = potassium concentration in μg/mg tissue wet weight

differences as compared to wet weight activity changes, but does not essentially alter the form of the curves

Discussion

In the dog a very rapid neurological development takes place during the first few weeks of life This fact makes longitudinal studies of the central effect of drugs difficult, since a uniform response cannot be expected in the different age groups In the present study we have tried partly to compensate for these difficulties by the simultaneous investigation of control animals with reproducible test procedures designed for the age group in question Because of the stereotype behaviour of the newborn puppies, the results obtained with this age group have to be interpreted with some caution However, two test parameters, i e righting and placing, reflexes could be assessed in all age groups In the newborn no effects of atropine on these two parameters were seen in contrast to the older dogs The three weeks old animals showed a rather well-differentiated behaviour permitting a considerably more accurate behavioural evaluation In this age group six out of seven animals showed an increased locomotor activity, while only four out of twelwe showed the other symptoms obtained in the CAS The animals showing the CAS appeared neurologically and behaviourally more mature than those who did not show this effect This finding and the fact that three out of four animals showed no symptoms even after 5 mg/kg suggest that there is a very rapid maturation of structures from which the CAS is elicited around three weeks of age It is interesting that at this age atropine just begins to influence the EEG in a manner similar to that seen in adult dogs (Roz

It thus seems justifiable to state that in the dog there is a profound change in the central effects of atropine during the first three to six weeks of life

Oxotremorine probably acts by direct stimulation of muscarinic receptors (see review by KARLÉN 1970), at least, this is the case with the isolated ileum preparation (COX & HECKER 1971) It seems to be generally accepted that the tremorogenic action of oxotremorine is a central effect, although the exact site of action is a matter of controversy However, most data point to a main site of action in the midbrain, while more rostral structures seem without importance in this respect (see review by JENDEN 1968, COX & POTKONJAK 1969) The reason for the absence of tremor in newborn animals is probably not due to a peripheral immaturity of the neuromuscular system, since these animals often show spontaneous tremor and it has been suggested that oxotremorine activates mechanisms normally involved in shivering (DECIMA & RAND 1965) Furthermore, in the rat there seems to be a correlation between the ontogeny of drug induced tremor for a series of drugs including oxotremorine, and the maturation of the different brain structures on which they act (HENDERSON & WOOLEY 1970) The finding that the central sensitivity to oxotremorine tremor developed earlier than the sensitivity to the behavioural effects of atropine suggests that these two symptoms are, to a great extent, elicited from different structures The general concept is that the brain matures in a caudal-rostral direction (see review by HIMWICH 1970) Thus, if oxotremorine tremor is elicited mainly from the midbrain, the CAS would mainly be elicited rostrally to this brain part Support for the theory of an immature cholinergic system in the brain at birth is also obtained from the increase with age in the activity of CAT and AChE, of which the former is probably the better cholinergic marker (HEBB 1956, BURT 1969)

The largest relative increase in CAT activity per gram wet weight from birth to adulthood was found in the caudate nucleus, while the cerebellum showed only a small increase, even preceded by a decrease, as has also been reported for other animals (HEBB 1956) According to several other parameters the cerebellum is more mature at birth than the caudate nucleus and the cerebral cortex (HIMWICH 1970) In this connection the twofold increase in cortical CAT activity might seem low However, it should be noted that the three ways of expressing relative enzyme activity in the present study i e per unit wet weight dry weight or potassium, probably underestimate neuronal increase in enzyme concentration The reasons for this are the relative increases with age in two components with a low enzyme content, i e myelin (FOX 1968) and intracellular volume attributable to the glial cells (VERNANDAKIS & WOODBURY 1965) The total enzyme activity of a structure has been used as an estimate of development by some investigators (BURT 1969) As seen in fig 2 there is a large rise in enzyme

activities in the different structures when expressed in this way, but there are also small differences in the course of the increase between the different structures Furthermore, except for the CAT activity in the caudate nucleus, the increases in total enzyme activities in the different structures are almost as large from 6 weeks of age to adulthood, as from birth to 6 weeks of age This however, does not correlate well with the fact that at six weeks of age the dog is neurologically and neurophysiologically almost mature (Fox 1968) There are a few other data indicating that cholinergic mechanisms are poorly developed in the dog cerebral cortex at birth ROZANOVA (1967) found a sharp increase in AChE content in the rostral midbrain in puppies around 3 weeks, i e the same age as that when the cortical EEG starts to respond to intravenous administration of ACh and atropine However, the AChE levels in the cortex were not determined

Biochemical and pharmacological data dealing with regional development of cholinergic mechanisms in the dog are thus rather scanty This is also true for other biochemical data in dog CNS (see review by AGRAWAL & HIMWICH 1970) Oxygen consumption *in vitro* in dogs is approximately doubled in the cortex and caudate nucleus and increases by only about 15 per cent in the cerebellum from one week of age to adulthood (HIMWICH & FAZEKAS 1941) The time course of these changes correlates with the increase found in CAT concentrations, except that the dips demonstrated at six weeks in the cortex and at three weeks in the cerebellum are not reflected The same also holds for glutamic acid, which increases about 2 to 3 fold in the cortex, caudate nucleus and cerebellum during postnatal maturation (DRAVID *et al* 1965) Regarding transmitter substances, only dopamine and noradrenaline have, to our knowledge, been investigated in dogs during development Dopamine increases about 5 fold in the caudate nucleus (AGRAWAL & HIMWICH 1970) but the time course is slower than for CAT Noradrenaline was found in comparatively low concentration in the midbrain and brain stem and not at all in the medial forebrain in 2–6 day old puppies The amounts rapidly increased and reached a maximum at 3–4 weeks of age and then declined toward adult values This time course approximately paralleled that of AChE which however, was already found in the forebrain at birth No telencephalic structures were investigated (ROZANOVA 1968) When interpreting these changes, the same factors must be taken into consideration as discussed above for the relative enzyme activities

Neuro anatomical, neurological, and behavioural development has, on the other hand, been extensively studied (see reviews by Fox 1966a & 1968) and a few such data will be briefly described Decerebration by transection of the midbrain just anterior to the pons had little or no effect on negative geotaxis, rooting and righting reflexes in one day old puppies In one week old puppies only the righting reflex was relatively unaffected, and in the six

week-old animals even this reflex was abolished by such transection, indicating an increasing influence of more rostral structures on these reflexes with age (Fox 1966b) These data should be viewed in relation to the lack of effect of atropine on these reflexes in newborn puppies in contrast to the influence in some three week-old animals Furthermore, atropine slowed the righting reflex in the older dogs but not in the newborn On the other hand the effect on the forelimbs of this transection, was the same in newborn and adult animals (Fox 1966b), while an increasing influence of atropine with age on this reflex was also observed The increasing effects of atropine on the other reflexes with an increasing influence by rostral structures suggests that atropine mainly acts on these higher levels If this assumption is correct, the increasing effect of the drug on the placing reflex would suggest a similar increase in the importance of rostral structures for this reflex too The effect of more rostral transections on the placing reflex has not, however, to our knowledge been studied

At five to six weeks of age, puppies are neurologically and neurophysiologically almost mature This is also true for cortical neuronal density, gross morphology and the biochemical parameters of the CNS investigated with the exception of myelin content The time course of this general brain maturation correlates well with the development of the central effects of atropine and the tremorogenic effect of oxotremorine It seems likely that these changes are due to a parallel maturation of the central cholinergic mechanisms, but the few data available do not allow a definite conclusion to be drawn

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REFERENCES

Adolph E F Ranges of heart rates and their regulations at various ages (rat) *Amer J Physiol* 1967 **212** 595–602

Adolph, E F Ontogeny of heart rate controls in hamster rat and guinea pig *Amer J Physiol* 1971 **220** 1886–1902

Agrawal H C & W A Himwich Amino acids proteins and monoamines of developing brain In *Developmental neurobiology* Ed W A Himwich Charles C. Thomas Springfield 1970

Albanus L Central and peripheral effects of anticholinergic compounds *Acta pharmacol et toxicol* 1970 **28** 305–327

Albanus, L., S-M Aquilonius, A Sundwall & B Winbladh The fate of intracerebroventricular injection of atropine and methylatropine in relation to their pharmacological effects *Acta pharmacol et toxicol* 1969, 27, 81–96

Albertoni, P Untersuchungen uber die Wirkung einiger Arzneimittel auf die Erregbarkeit des Grosshirns nebst Beiträgen zur Therapie der Epilepsie *Arch Exp Path Pharmacol* 1882, XV, 248–288

Baklavadzjan, O G & F A Adamjan [Pharmacological analysis of primary responses from cerebral cortex during the ontogenesis] *Fiziol Zh SSSR I M Sechenova* 1964, 50, 145–152 (in Russian)

Boreus, L O & D M McMurphy Ontogenetic development of cholinergic receptor function in guinea pig ileum *Acta Physiol Scand* 1971, 81, 143–144

Burt, A Acetylcholinesterase and choline acetyltransferase activity in the developing chick spinal cord *J Exp Zool* 1969, 169, 167–212

Campbell, B A, L D Lytle & H C Fibiger Ontogeny of adrenergic arousal and cholinergic inhibitory mechanisms in the rat *Science* 1969, 166, 635–637

Cox, B & S E Hecker Investigation of the action of oxotremorine on the guinea pig isolated ileum preparation *Brit J Pharmacol* 1971, 41, 19–25

Cox, B & D Potkonjak An investigation of the tremorgenic effects of oxotremorine and tremorine after stereotaxic injection into rat brain *Int J Neuropharmacol* 1969, 8, 291–297

Decima, E E & R W Rand Oxotremorine induced tremor in the decorticated cat *Int J Neuropharmacol* 1965, 4, 139–148

Dravid, A R, W A Himwich & J M Davis Some free amino acids in dog brain during development *J Neurochem* 1965, 12, 901–906

Ellman, G L, K D Courtney, V Andres Jr & R M Featherstone A new and rapid colorimetric determination of acetylcholinesterase activity *Biochem Pharmacol* 1961, 7, 88–95

Eriksson, K H & B Winbladh Choroid plexus uptake of atropine and methylatropine *in vitro* *Acta Physiol Scand* 1971, 83 300–308

Fonnum, F Isolation of choline esters from aqueous solutions by extraction with sodium tetraphenyl boron in organic solvents *Biochem J* 1969, 113 291–298

Fox, M W The ontogeny of behaviour and neurologic responses in the dog *J Animal Behaviour* 1964, 12, 301–310

Fox, M W *Canine pediatrics* Charles C Thomas Springfield 1966a.

Fox M W The clinical significance of age differences in the effects of decerebration and spinal cord transection in the dog *J Small anim Pract* 1966b 7, 91–98

Fox, M W Multidiscipline studies of the postnatal development of the CNS and behaviour of the dog In *Ontogenesis of the brain* Ed L Jilek and S Trojan Charles University, Prague 1968

Hebb, C O Choline acetylase in the developing nervous system of the rabbit and guinea pig *J Physiol* 1956 133, 566–570

Henderson G L & D E Wooley Ontogenesis of drug induced tremor in the rat *J Pharmacol Exp Therap* 1970, 175, 113–120

Himwich, H E Historical review in *Developmental neurobiology* Ed W A Himwich Charles C Thomas, Springfield 1970

Himwich, H E & J F Fazekas Comparative studies of the metabolism of the brain of infant and adult dogs *Amer J Physiol* 1941, 132, 454–459

Jenden D J Testing of drugs for therapeutic potential in Parkinson's disease In *Selected pharmacological testing methods* Ed A Burger Vol 3 Marcel Dekker New York 1968

Karlen, B Chemical and biological studies of tremorine, oxotremorine and oxotremorine antagonism *Acta Pharm Suecica* 1970 7, 169–200

Kovach, J K Effects of autonomic drugs on imprinting *J Comp Physiol Psychol* 1964, 57, 183–187

Lhoták, K Über die Ursachen der sinkenden Pulsfrequenz bei wachsenden Hunden *Arch Ges Physiol* 1911, 141, 514–526

Mannervik, B & B Sörbo Inhibition of choline acetyltransferase from bovine caudate nucleus by sulfhydryl reagents and reactivation of the inhibited enzyme *Biochem Pharmacol* 1970 19, 2509–2516

McGeer, P L. & E G McGeer Caudate nucleus choline acetylase Effects of lesions and age *Fed Proc* 1971, *30* (3), 1085

McMurphy, D M & L O Boreus Studies on the pharmacology of the perfused human fetal ductus arteriosus *Amer J Obstet Gynecol* 1971, 109, 937–942

Ostle B *Statistics in research* Iowa State College Press Iowa 1954

Rozanova V K [On the role of cholinergic mechanisms in the activation and inhibition of the cortical electric activity in dogs during the ontogenesis] *Zh Evolut biokim fiziol* 1967, 3 48–53 (in Russian)

Rozanova, V D *Essays on experimental developmental pharmacology* Leningrad 1968 (in Russian)

Schlossman H A The relationship between age and the action of atropine and morphine *J Pharmacol Exp Therap* 1937, 60, 14 31

Schuberth, J & B Sorbo Measurement of choline acetylase In *Methods of biochemical analysis*, vol 19 Ed D Glick J Wiley 1971

Vernandakis, A & D M Woodbury Cellular and extracellular spaces in developing rat brain *Arch Neurol* 1965, 12, 284–293

Winbladh, B Uptake of atropine and methylatropine by immature choroid plexus *in vitro* *Acta Physiol Scand* 1972 84, 109–114

Winbladh B Fate of atropine in the puppy *Acta pharmacol et toxicol* 1973, 32, 46–64

Yannet, H & D C Darrow The effect of growth on the distribution of water and electrolytes in brain liver, and muscle *J Biol Chem* 1938 123 295–305

Acta pharmacol et toxicol 1973, 32, 83–87

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Deacetylation of Phenylthioacetate in the Presence of Oximes

By

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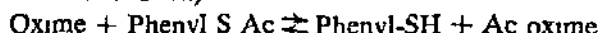
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Abstract In the presence of oximes phenylthioacetate is deacetylated and acetylated oximes formed By measuring the velocity of this transacetylation reaction for a number of oximes *in vitro* the slopes are found to differ in a way which resembles the variance in the potency of the oximes *in vivo* as reactivators for cholinesterases inhibited by alkyl organophosphorous compounds The observations thus support the hypothesis that oximes as acetyl acceptors might have an effect by inhibition of the synthesis of acetylcholine due to deacetylation of acetyl-coenzyme A

Key words Oximes – phenylthioacetate – transacetylation velocity

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Because of the greater lipoid solubility phenylthioacetate was introduced as a substrate for the histochemical demonstration of esterases by BOOT & METCALF (1970) As thiocholine esters (KOELLE 1957, BERGNER & O NEILL 1958) phenylthioacetate reacts with oxime in a transacetylation following this reaction (O NEILL *et al* 1961)



Oxime + Phenyl S Ac $\rightleftarrows$ Phenyl-SH + Ac oxime

Based on this *in vitro* and other observations these investigators put forward the theory that the therapeutic value of oximes does not solely depend on their ability to reactivate the cholinesterase inhibited by alkyl organo phosphorous compounds but that it is also dependent on ability to inhibit the synthesis of acetylcholine Since they are acetyl acceptors they might inhibit this synthesis by deacetylating the necessary acetyl coenzyme A

By measuring the velocity of the transacetylation described above, it might be possible to illustrate this hypothetical part of the antidote effect that oximes have in poisoning with alkyl organophosphorous compounds Therefore, the velocity of the transacetylation reaction with a number of oximes which are or have been used in the treatment of such poisoning has been measured *in vitro*

Table 1

Oximes	ΔA / conc oxime	correl coeff
Toxogonin, bis (4 formyl N methylpyridinium oxime) ether dichloride	1240	0 994
TMB-4, (N N Trimethylene bis pyridine-4 aldoxime bromide)	1150	0 996
Pralidoxime iodide, P 2 AM J, (Pyridine-2 aldoxime methiodide)	500	0 998
Pralidoxime chloride, P 2 AM Cl, Pyridine 2 aldoxime methochloride)	490	0 991
Pralidoxime methansulphonate, P2S, (Pyridine 2 aldoxime methylmethanesulphonate)	420	0 999
Hydroxylamine	140	0 822
DAM, Diacetylmonoxime	43	0 990
Pyridine 2 aldoxime	15	0 998

Material and Methods

Reagents

Buffer solutions according to Ph Nord Ed Danica 63, vol I
Hydrogen ion buffer pH 8 0
Phosphate buffer pH 7 0
DTNB reagent 5 5 dithio-bis 2 nitrobenzoic acid 39 6 mg
Sodium bicarbonate 15 0 mg
Phosphate buffer pH 7 0 to 10 0 ml

Phenylthioacetate was synthesized by heating 60 mmol of thiophenol and 480 mmol of acetic anhydride under reflux in a steam bath for 10 minutes After the reaction mixture had cooled 100 ml of ice water was added through the cooler and the mixture was extracted with ethyl ether The extract was washed twice with a 2 N sodium hydroxide solution mixed with crushed ice, and twice with icecold water The ether solution was dried with anhydrous sodium sulphate and the ether was evaporated The remaining phenylthioacetate was destilled in vacuum bp 109°/13 mm Hg (litt 110°/13 mm Hg)

The oximes used are shown in table 1

Methods

The reaction was followed by measuring the amount of released thiophenol spectrophotometrically as described by ELLMAN *et al* (1961) The spectrophotometer used was a Beckman DB connected to a 10" logarithmic Beckman recorder and the alteration in extinction at a fixed wavelength (λ = 412 nm) was registrated as a function of time All experiments were carried out at 30° in thermostated cuvettes

In the part of the investigation dealing with the influence of the oxime concentration on the velocity of the transacetylation the initial concentration of phenylthioacetate was $1\ 5 \times 10^{-4}$ M

The change in absorbance (Δ A) during the second minute after addition of oxime

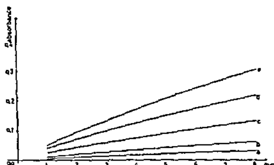


Fig 1 The reaction between phenylthioacetate and various concentration of pyridine 2 aldoxime methyl methansulphonate (P 2 S) as a function of time Abscissa Time in minutes Ordinate absorbance
The recorder was started 1 min after mixing the reagents Initial concentration of phenylthioacetate was 1.5×10^{-4} M The concentration of P 2-S was a 10^{-5} b 2×10^{-5}, c 4×10^{-5}, d 7×10^{-5}, e 10^{-4} M

was used as a measure of the reaction velocity In one series of experiments on the investigation of the influence of the concentration of phenylthioacetate on the velocity of the reaction the concentration of this compound was varied while the concentration of the oxime in this case pralidoxime methylmethansulphonate (P2S) was kept constant at 4×10^{-5} M

Results

Fig 1 shows the curves obtained by registering the deacetylation of phenylthioacetate at different concentrations of the oxime P2S The curves obtained are not straight lines

The change in absorbance during the second minute (△ A) was taken as a measure of the decomposition rate of phenylthioacetate

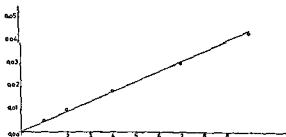


Fig 2 The change in absorbance (ΔA) during the second minute as a function of P 2-S concentration Abscissa Mol concentration of P 2-S multiplied by 10^5 Ordinate ΔA change in absorbance during the second minute.

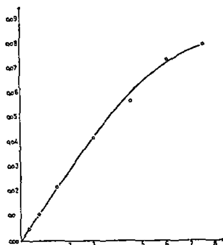


Fig 3 The reaction between P 2-S and phenylthioacetate as a function of phenylthioacetate concentration Initial concentration of P 2 S was 4×10^{-5} M Abscissa Mol concentration of phenylthioacetate multiplied by 10^4 Ordinate Δ A change in absorbance during the second minute

Fig 2 shows the dependence between $\triangle$ A and the concentration of oximes in this case P2S This curve is a straight line through 0 0 and with a slope of 420

The slopes $\frac{\triangle A}{\text{conc oxime}}$ were calculated in a similar way for the series of oximes listed in table 1, which also gives the corresponding correlation coefficients

Fig 3 shows the influence on the reaction velocity of phenylthioacetate determined at different concentrations It appears that a strong deviation from a straight line occurs at concentrations higher than 4×10^{-4} M

Discussion

The slopes found are listed in table 1 in order of decreasing values It is worth noting that the order obtained for the oximes corresponds to that generally accepted for the potency of the oximes as reactivators for cholinesterase inhibited by alkyl organophosphorous compounds

According to this finding the transacetylation rates not only seem to give an indication of the ability of oximes to inhibit the synthesis of acetylcholine but also give a picture of their therapeutic value as re-activators of cholinesterases inhibited by alkyl organophosphorous compounds

As the synthesis of phenylthioacetate is easy and cheap, and the compound

is stable, non hygroscopic and easy to handle the compound also with advantage can replace acetylthiocholine in the method for the determination of oximes described by KARLOG & PETERSEN (1963)

REFERENCES

Bergner, A D & J J O'Neill A modification of the Koelle technique for use with oximes *J Histochem Cytochem* 1958, 6 72–74

Boot, G M & R L Metcalf Phenylthioacetate A usefull substrate of the histochemical and colorimetric detection of cholinesterase *Science* 1970 **170** 455–457

Ellman G L, K D Courtney, Jr V Anders & R M Featherstone A new and rapid colometric determination of acetylcholinesterase activity *Biochem Pharmacol* 1961, 7, 88–93

Karlog O & H E Petersen The influence of oximes on the acetylthiocholine hydrolysis rate *Biochem Pharmacol* 1963, **12** 590–591

Koelle, G B Histochemical demonstration of reactivation of acetylcholinesterase *in vivo* *Science* 1957, **125** 1195–1196

O Neill, J J, H Kohl & J Epstein The reaction between pyridine-4 aldoxime and methiodide thioesters A model for enzymic transacetylation *Biochem Pharmacol* 1961, 8 399–407

Acta pharmacol et toxicol 1973, 32, 88–96

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The Enzymatic Hydrolysis of a Series of Aminoacylanilides in Relation to their Nerve Blocking Effect and Toxicity

By

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Abstract Evidence has been adduced that in a series of aminoacylanilides the extent to which enzymatic hydrolysis is effected by liver amidases is dependent on the steric positions of substituents The significance of hydrolysis for the detoxification of aminoacylanilides was examined by repeated administration of the compounds at a slow rate to rabbits Compounds that were rapidly broken down by amidases displayed a lower cumulative toxicity than those resistent to the enzymes On the other hand, there was no evident correlation between the enzymatic activity and the acute intravenous or subcutaneous toxicity (LD50 values) following rapid injection of large doses The toxic potency in this case was directly releated to the blocking action on the isolated nerve In the case of *o* toluidine derivatives, there was a close correlation between the rate of hydrolysis *in vitro* and the formation of methaemoglobin With 2,6-xylidine derivatives, no significant formation of methaemoglobin was observed in spite of rapid hydrolysis

Key-words Local anaesthetics – lidocaine – mepivacaine – prilocaine – aminoacylanilides

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The systemic toxicity of local anaesthetic agents is diminished by rapid metabolism Most of these agents are esters or amides and these bonds are sites susceptible to enzymatic hydrolysis However, the observation by HOLLUNGER (1960) that lidocaine (diethylaminoaceto-2,6 xylidide, xylocaine®) was resistant to liver amidases whereas it underwent oxidative de-ethylation by liver microsomes *in vitro* suggest the action of processes other than hydrolysis in the enzymatic breakdown of this type of agents On the other hand, another local anaesthetic agent of the amide type, prilocaine (α-propylamino-2-methylpropionanilide, citanest®) is rapidly hydrolyzed by liver amidases (GEDDES 1965, AKERMAN *et al* 1966a)

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The present study was undertaken to ascertain the factors determining the rate of hydrolysis *in vitro* of a series of aminoacylanilides related to lidocaine, and to analyze the relationship between such factors and the acute toxicity and nerve-blocking action of these drugs The formation of methaemoglobin in the cat was also investigated

Material and Methods

Hydrolysis

In the study of the rate of metabolism *in vitro* tritiated compounds were used The compounds were labelled by the method of YAVORSKY & GORIN (1962), in which the hydrogen atoms in the benzene nucleus are replaced by tritium atoms

The rate of hydrolysis of the compounds by mouse liver amidases *in vitro* was determined by a method described elsewhere (AKERMAN *et al* 1966a) Liver homogenate was prepared in 10 volumes of ice cold 0 1 N trishydroxymethylaminomethane (Tris) buffer, pH 8.5 The incubation mixture contained 0 5 ml of homogenate and 0 4 µmol of the tritiated compound The final volume was 1 0 ml The incubation was performed at 37° for 10 or 30 min in a metabolic shaker The enzyme reaction was terminated by adding 1 ml of 10 per cent trichloroacetic acid Each assay was made in duplicate or triplicate After being made alkaline with 1 N sodium hydroxide, the intact local anaesthetic agent and the aniline derivative formed were extracted with 20 ml of heptane by shaking for 20 min The aniline derivatives in the extract were assayed by shaking 10 ml of the heptane extract with the same volume of 1 N acetate buffer with pH 4 0 for *o*-toluidine and 2,6 xylidine and with pH 5 0 for aniline At these pH levels 67 per cent of 2,6 xylidine, 32 per cent of *o* toluidine and 36 per cent of aniline remained in the heptane phase whereas the local anaesthetic agents were almost completely transferred into the water phase. The amount of aniline derivative formed was calculated and was expressed in micromoles per gramme per min

The rate of hydrolysis of the tritiated compounds by slices of cat liver was determined by a similar technique The incubation of 250 mg of liver slices was performed with 0 4 µmol of the tritiated compound in 3 ml of Krebs-Henseleit s solution by shaking at 37° for 10 or 30 min in a metabolic shaker with an atmosphere of oxygen containing 6 5 per cent of carbon dioxide The reaction was arrested by adding 5 ml of 2 per cent trichloroacetic acid After homogenization and making alkaline with 1 N NaOH the local anaesthetic agent and the aniline derivatives formed were extracted with 20 ml of heptane and the amount of the aniline derivative formed was determined as above

The radioactivity in the heptane extracts was determined in a liquid scintillation system (Packard Tri-Carb) using 0 3 per cent of 3 4-diphenyloxazole (PPO) and 0 01 per cent of β bis [2 (phenyloxazolyl)] benzene (POPOP) in toluene as the scintillation liquid

Toxicity

The LD50 values for intravenous and subcutaneous injection were determined in male albino mice (18–22 g) of the same strain (NMRI) 10 animals were injected for each of 6 dose levels The local anaesthetics were injected as 0 2 per cent solutions in isotonic saline (pH 5 8–6 2) except in a few cases of the less toxic agents, when a concentration of 0 4 per cent was used so as to keep the volume injected within 0 5 ml In the subcutaneous series the concentrations varied from 2 per cent to, in a few cases

8 per cent The LD50 was determined by the method of LITCHFIELD & WILCOXON (1949)

In another series of experiments the duration of the loss of the righting reflex (LRR) was determined in rabbits following repeated slow injections into a marginal ear vein every 15 min of a 2 per cent solution (pH 6 8–7 2) The dose used was such as to produce a loss of the righting reflex lasting less than 1 min (mean for 5 animals) 10 consecutive injections were made in each animal

Nerve blocking action in vitro

For determining the blocking of impulse conduction by the method of MAURO *et al* (1948) and ÅSTRÖM & PERSSON (1961) isolated sciatic nerves of the frog (Rana esculenta) were used The fall in the action potential (A spike) was recorded after bathing a small segment of the nerve for 5 min in a 5 mM solution of the compound (Tasaki Ringer, pH 7 4) To test the specimen each nerve was blocked with the same concentration of lidocaine before application of the test solution The action of the compounds was expressed in relation to that of lidocaine on the same nerve

Formation of methaemoglobin

Healthy cats of both sexes (2 5–3 5 kg) were anaesthetized with pentobarbitone sodium (mebumalum NFN) 30 mg/kg intraperitoneally, 20 mg/kg of the compound dissolved in 2 per cent solution (pH 6 8–7.2) was infused at a rate of 0 1 ml/min into the femoral vein by a Palmer slow injection apparatus At regular intervals for up to 6 hours after the infusion was started 1 5 ml blood samples were withdrawn Spectrophotometric analysis for methaemoglobin was performed as described by EVELYN & MALLOY (1938)

Results

Hydrolysis by mouse liver amidases in vitro

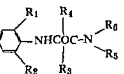
The relation between the rate of hydrolysis by mouse liver amidases *in vitro* and the chemical structure of a number of anilides, *o*-toluidides and 2,6-xylidides was examined The results are presented in table 1

The rate of hydrolysis generally decreased with the number of substituents around the amide linkage The substitution that had the greatest influence on the enzymatic hydrolysis appears to be located at the neighbouring carbon in the side chain One methyl group in this position reduced the rate of hydrolysis (compare V with II, VI with III and XIII with III) Compounds with 2 methyl groups in this position were only slightly or not at all hydrolyzed by the amidases (XVII and XVIII) In the xylidide series the tertiary amines (VII–X) were slowly hydrolyzed by the enzymes, in contrast to the tertiary toluidide derivatives (XIX, XX) An increase in the size of the substituent on the nitrogen increased the rate of hydrolysis of the secondary amines among the xylidide (I-IV) and the toluidide derivatives (XII–XVI)

Toxicity in mice and rabbits

A conventional study of acute toxicity in mice disclosed no significant correlation between the intravenous ($r = 0\,16$, $P > 0\,05$) or subcutaneous

Enzymatic hydrolysis, acute toxicity and nerve blocking action of a series of aminoacylanilide derivatives



Compound	R_1	R_2	R_3	R_4	R_5		Rate of hydrolysis by mouse liver homogenate $\mu mol\ g^{-1} min^{-1}$ [a]	Acute toxicity in mice LD50 mmol/kg i v	Acute toxicity in mice LD50 mmol/kg s c	Blocking action on nervous conduction in isolated frog sciatic nerve [b] Lidocaine (*VII*) = 1.0
I	CH_3	CH_3	H	H	H	CH_3	0.04	0.19	2.80	0.1
II	CH_3	CH_3	H	H	H	C_2H_5	0.12	0.22	1.44	0.2
III	CH_3	CH_3	H	H	H	C_3H_7 (-n)	0.53	0.11	0.95	0.9
IV	CH_3	CH_3	H	H	H	C_3H_7 (-iso)	0.39	0.18	1.87	0.5
V	CH_3	CH_3	CH_3	H	H	C_2H_5	< 0.01	0.13	1.71	0.7
VI	CH_3	CH_3	CH_3	H	H	C_3H_7 (-n)	0.02	0.06	1.00	1.0
VII	CH_3	CH_3	H	H	C_2H_5	C_2H_5	< 0.01	0.09	1.00	1.0
VIII	CH_3	CH_3	CH_3	H	CH_3	CH_3	< 0.01	0.09	0.92	0.9
IX	CH_3	CH_3	CH_3	H	C_2H_5	C_2H_5	< 0.01	0.06	0.81	1.1
X	CH_3	CH_3	H	-$(CH_2)_4$-		CH_3	< 0.01	0.10	0.95	0.6
XI	CH_3	H	H	H	H	CH_3	0.24	0.35	4.71	0.1
XII	*CH_3*	*H*	*CH_3*	*H*	*H*	C_2H_5	0.06	0.20	2.97	0.2
XIII	CH_3	H	CH_3	H	H	C_3H_7 (-n)	0.07	0.16	2.67	0.6
XIV	CH_3	H	CH_3	H	H	C_3H_7 (-iso)	0.01	0.17	2.84	0.5
XV	CH_3	H	CH_3	H	H	C_4H_9 (-n)	0.21	0.07	1.55	1.0
XVI	CH_3	H	CH_3	H	H	C_4H_9 (-iso)	0.39	0.07	2.03	1.0
XVII	CH_3	H	CH_3	CH_3	H	C_3H_7 (-n)	< 0.01	0.09	2.88	0.9
XVIII	CH_3	H	CH_3	CH_3	H	C_6H_{13}	< 0.01	0.06	2.16	1.0
XIX	CH_3	H	H	H	C_2H_5	C_2H_5	0.08	0.14	5.84	0.2
XX	CH_3	H	CH_3	H	C_2H_5	C_2H_5	0.05	0.15	4.17	0.7
XXI	H	H	CH_3	H	H	C_3H_7	0.20	0.17	1.90	0.6

[a] *Incubation mixture* 0.5 ml of mouse liver homogenate (1:10 in 0.1 N tris buffer, pH 8.5) and 0.4 μmol of the tritiated compound. Final volume 1.0 ml. The incubation was performed for 10 or 30 min. at 37°. The aniline derivative formed was analyzed as described under *Methods*.

[b] Decrease in amplitude of action potential on incubation for 5 min with 5.0 mM solutions (Tasaki Ringer, pH 7.4).

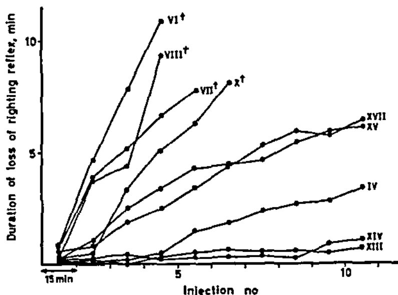


Fig 1 Cumulative toxicity in rabbits Mean duration ($n=5$) of loss of righting reflex following repeated slow intravenous injections each at 15 min interval The dose was chosen so as to produce a reflex loss of less than 1 min. At the first injection for compound VI and VIII it was 6 mg/kg for VII X XV and XVII 8 mg/kg for IV, 10 mg/kg, and for XIII and XIV, 12 mg/kg

+ Denotes that the experiment was discontinued because the rabbit died

($r=0.06$, $P>0.05$) toxicity (LD50 values) of the compounds and the rate of enzymatic hydrolysis *in vitro* (table 1) Slow repeated intravenous injections of toxic but sub-lethal doses yielded different results, however, the toxicity would then be expected to be influenced by the rate of metabolism to a greater extent than when a large, single dose is injected rapidly The duration of the loss of the righting reflex generally increased inversely with the rate of hydrolysis by amidases *in vitro* (fig 1, e g VI, VII, XV and IV) In exceptional cases, as for example in XIV, there was no cumulative toxicity in spite of resistance to amidase

Blocking action in isolated sciatic nerve of the frog

The ability of the compounds to block the propagation of impulses in the isolated nerve in relation to that of lidocaine is shown in table 1 The nerve-blocking action *in vitro* was correlated directly to the intravenous toxicity ($r=0.86$, $P<0.001$) and fairly directly to the subcutaneous toxicity ($r=0.60$, $0.01>P>0.001$) as indicated by the LD50 values (fig 2)

Hydrolysis by cat liver and methaemoglobin formation in vivo

There was a fairly close relation between the rate at which *o*-toluidide

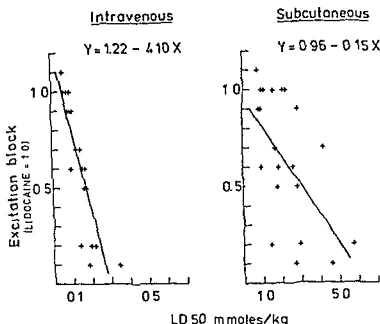


Fig 2 Relationship between the intravenous or subcutaneous toxicities and the nerve blocking action The values are taken from table 1

derivatives were hydrolyzed by cat liver slices and the rate of methaemoglobin formation *in vivo* (fig 3) The peak levels of methaemoglobin also tended to be higher for rapidly hydrolyzed compounds, such as XV, XIX, than for the more slowly catabolized ones such as XX The 2,6-xylidide derivatives did not form methaemoglobinaemia to any significant extent even when the agent (IV) was rapidly hydrolyzed The only anilide derivative studied (XXI) was one of the most active compounds tested

Discussion

Although the substrate specifity of the liver amidases is low the differences in the rate of the hydrolysis of the aminoacylanilides investigated were probably due mainly to a different strength of the steric hindrance of the substituents This is indicated by, for instance, the observation that both the tertiary xylidide derivatives and the 2 2 dimethyl-substituted agents were resistant to hydrolysis On the other hand, in the secondary amine a large substituent on the amine nitrogen appeared to induce a higher affinity for the enzyme, since these compounds were more rapidly hydrolyzed than those with a small substituent in this position

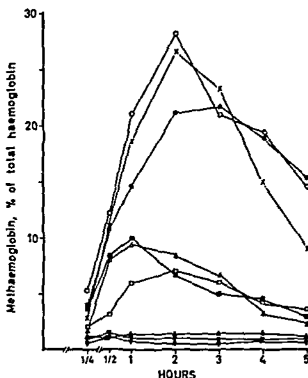


Fig 3 Formation of methaemoglobin in the anaesthetized cat, expressed as a percentage of the total haemoglobin

The compounds (20 mg/kg) were infused intravenously over a period of 20 min Each compound was used in 3 cats, except for VII, which was used in 8 cats The greatest level of methaemoglobinaemia observed in the control specimens was 1 3 per cent

Compound	XIX	XV	XIII	XXI	XX	XIV	XVII	IV	VII
Notation	○	●	■	×	□	△	▲	▽	▼
Rate of hydrolysis by slices of cat liver *in vitro* nmol g^{-1} min^{-1}	59	46	42	25	4	10	0	31	20

There was no significant correlation between the intravenous or subcutaneous LD50 values of the compounds and the rate of hydrolysis by amidases *in vitro* It is probable that when a large, single dose is injected rapidly as in the LD50 determinations, the toxicity is only slightly modified by a high rate of metabolism In this case the toxicity would seem to be more closely related to the intrinsic action on the central nervous system The close correlation between the intravenous LD50 values and the nerve-blocking action *in vitro* observed in this study is consistent with the results reported by ÅKERMAN *et al* (1966a) in an earlier study

Under conditions other than those associated with the rapid injection of large doses in small animals, the toxicity of local anaesthetic agents is de-

termined by several factors in addition to the toxic potency *per se* It would be expected therefore that the longer period of administration the more significant would be the rate of metabolism The importance of the rate of hydrolysis for the de toxification of the aminoacylanilides was demonstrated in the experiments with slow intravenous injections into rabbits Compounds rapidly hydrolyzed by amidases *in vitro* were much better tolerated when administered by repeated injections at a slow rate than were those resistant to the enzymes The latter are probably metabolized, at least initially, otherwise than by hydrolysis (AKERMAN *et al* 1966a, HANSSON *et al* 1965) It would seem that rapid catabolism by hydrolysis reduces the toxicity more than other forms of bio transformation, a result that may be of practical importance Compound XIII (prilocaine) for example, was rapidly broken down by amidases and well tolerated at repeated injections, whereas compound X (mepivacaine) was almost unaffected and exhibited a marked cumulative toxicity when administered repeatedly to rabbits in the same dose as the former compound The cumulative effect of mepivacaine has been established in other studies (TRUANT & WIEDLING 1958, HENN 1960, MOORE *et al* 1968)

Methaemoglobinaemia is induced by a number of aromatic amides (HARRIS 1963, FUJIMORI *et al* 1964, HJELM & HOLMDAHL 1965) The results of the study in the cat indicate that all the *o*-toluidine derivatives that can be hydrolyzed are capable of forming methaemoglobin The rate at which methaemoglobin is formed was closely correlated with the rate of hydrolysis obtained with liver slices *in vitro* The findings are consistent with the observations of BRODIE & AXELROD (1949), KIESE (1965) and AKERMAN *et al* (1966b) that methaemoglobin formation is associated with the metabolic formation of aromatic amines or, more probably, their metabolites, following the enzymatic hydrolysis of the compounds The only aniline derivative used in the present study was one of the most active methaemoglobin inducers, while in the series of 2,6-xylilides no formation was observed in spite of rapid hydrolysis of some of the compounds This is in close agreement with the result reported by MCLEAN *et al* (1967) that 2 6 xylidine *per se* induced the formation of methaemoglobin to a far less extent than aniline

REFERENCES

Akerman B A Åstrom S Ross & A Telč Studies on the absorption distribution and metabolism of labelled prilocaine and lidocaine in some animal species *Acta pharmacol et toxicol* 1966a 24 389–403

Akerman B S A Petersson & P Wistrand Methemoglobin forming metabol tes of prilocaine *III International Pharmacological Congress Sao Paulo Bra il* 1966b 237

Astrom, A & N H Persson Some pharmacological properties of *o* methyl α propyl amino propionanilide a new local anaesthetic *Brit J Pharmacol Chemotherap* 1961, **16**, 32–44

Brodie, B B & J Axelrod The fate of acetophenetidine (phenacetin) in man and methods for the estimation of acetophenetidine and its metabolites in biological material *J Pharmacol Exp Therap* 1949, **97**, 58–67

Evelyn, K A & H T Malloy Microdetermination of oxyhemoglobin methemoglobin and sulfhemoglobin in a single sample of blood *J Biol Chem* 1938, **126**, 655–662

Fujimori, M K Nishimura & E Goto Methemoglobinemia due to local anesthetics (a preliminary report) *Far East J of Anesthesia* 1964, **4** 22–30

Geddes I C Studies of the metabolism of citanest C14 *Acta Anaesth Scand* 1965, Suppl XVI, 37–44

Hansson, E, P Hoffmann & L Kristerson Fate of mepivacaine in the body II. Excretion and biotransformation *Acta pharmacol et toxicol* 1965, **22**, 213 223

Harris J W The red cell Harward University Press, Massachusetts, 1963

Henn, F Determination of the toxicological and pharmacological properties of carbocaine, lidocaine and procaine by means of simultaneous experiments *Acta Anaesth Scand* 1960, **4**, 125–154

Hjelm, M & M H son Holmdahl Clinical chemistry of prilocaine and clinical evaluation of methaemoglobinaemia induced by this agent *Acta Anaesth Scand* 1965, Suppl XVI 161–170

Hollunger, G On the metabolism of lidocaine II The biotransformation of lidocaine *Acta pharmacol et toxicol* 1960, **17**, 365–373

Kiese, M Relationship of drug metabolism to methemoglobin formation *Ann N Y Acad Sci* 1965, **123**, 141–155

Litchfield Jr, J T & F Wilcoxon A simplified method of evaluating dose effect experiments *J Pharmacol Exp Therap* 1949, **96**, 99–113

Mauro A A P Truant & E L MacCawley Electrophysiological methodology for studies in neuropharmacology *Yale J Biol Med* 1948 **21**, 113–120

McLean S B P Murphy, G A Starmer & J Thomas Methaemoglobin formation induced by aromatic amines and amides *J Pharm Pharmacol* 1967, **19**, 146–154

Moore D C, P A Bagdi, L D Bridenbaugh & P O Bridenbaugh Accumulation of mepivacaine hydrochloride during caudal block *Anesthesiology* 1968, **29**, 585–588

Truant, A P & S Wiedling A contribution to the pharmacological and toxicological evaluation of a new local anaesthetic, d l N methylpipecolyl 2,6 xylidide *Acta Chir Scand* 1958 **116**, 351–361

Yavorsky P M & E Gorin New reagent for labelling organic compounds with tritium *J Amer Chem Soc* 1962, **84**, 1071–1072

Acta pharmacol et toxicol 1973, 32, 97–111

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Differing Local Anaesthetic Potency and Toxicity of the Enantiomers of N-n-butyl-3-piperidyl 2′-chloro-6′-methylcarbanilate: A New Local Anaesthetic

By

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Abstract The R(+) form of N n butyl 3 piperidyl 2 -chloro-6 methylcarbanilate (HS 37) was a more potent local anaesthetic than the S(−) form in various types of major nerve block, in infiltration anaesthesia and after topical application (guinea pigs, rabbits) The potency ratio varied with the method of administration and was between 2 and 3 in most of the tests A similar difference in blocking activity was obtained in rate and equilibrium blocks of conduction in sheathed and de sheathed sciatic nerve (frogs) The effect of the racemate (infiltration frog nerve) was intermediate to that of the enantiomers The R(+) form was more toxic than the S(−)-form both intravenously (mice, rabbits) and subcutaneously (mice) Nerve blocking and toxic potencies of the enantiomers were positively correlated with ratios of about the same order of magnitude The findings may indicate some stereo selectivity of action of the enantiomers in the excitable membranes rather than differences in concentration in the biophase

Key words Local anaesthetics – enantiomers – N n butyl 3 piperidyl 2 -chloro 6 methylcarbanilate

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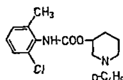
Recent studies have established different potencies of enantiomers of local anaesthetics such as prilocaine (ÅKERMAN *et al* 1967), bupivacaine (LUDUENA 1969), and mepivacaine (ADLER *et al* 1969) One possible explanation for the differences for these pairs was differences in absorption from the sites of application, resulting in differences in drug availability in the biophase Previous findings suggest that one factor responsible for the variance in potency of enantiomers of compounds with a local anaesthetic effect is stereo-sensitivity in the actual mechanism for block of excitation (ÅKERMAN *et al* 1967 & 1969, SCHONENBERGER *et al* 1967) Synthesis of a new potent local anaesthetic N-n butyl 3-piperidyl 2′-chloro-6′-methylcarbanilate, HS 37 (NILSSON *et al* 1971) and resolution of the racemate into its enantiomers (SIEVERTSSON *et al* 1972) made it possible to elucidate further

the role of steric factors in the action of local anaesthetics In the present report the R(+)- and S(–)- forms of HS 37 have been compared in a num ber of *in vivo* and *in vitro* tests for block of nervous conduction

Material and Methods

Drugs

The compounds used were the bases of the racemate and the enantiomers of N n butyl 3 piperidyl 2 chloro-6 methylcarbanilate, HS 37 (NILSSON *et al* 1971), prepared at the Research and Development Laboratories, Astra Lakemedel AB Sweden According to SIEVERTSSON *et al* (1972) the enantiomers have the following configuration R(+)- HS 37, S(–) HS 37



For the experiments *in vivo* the compounds were dissolved in saline (0 85 %) Unless otherwise stated the pH of the solutions without adrenaline was 5 6–6 5 and 3 7–4.5 for the solutions with adrenaline (bitartrate) A modified Tasaki Ringer solution of the composition NaCl 111 2 mM, KCl 1 88 mM $CaCl_2$ 1 08 mM $NaHCO_3$ 2 38 mM and NaH_2PO_4 0 06 mM was used as solvent for the compounds in the isolated nerve tests The pH was adjusted to 7 4 with the exception of one separate study In comparing solutions of the two forms of equal strength care was taken to ensure that the solutions were of the same pH

In vivo methods

Guinea pigs (300–350 g) of both sexes were used for sciatic nerve and plexus brachialis block peridural anaesthesia and for the intracutaneous wheal and tracheo-bronchial anaesthesia tests

Sciatic nerve block was produced by the injection of 0 2 ml of the solutions into the space surrounding the nerve at the hip (SHACKELL 1935) The incidence of block time of onset and recovery from motor paralysis (dragging the leg) and flexion reflex block (no withdrawal response to manual pressure on the foot) were recorded For *block of the plexus brachialis* onset time frequency and duration of motor paralysis were recorded after the injection of 0.2 ml of the solutions With few exceptions solutions of the enan tiomers of the same concentration were tested in the same animal but on different legs in these two tests The second test was carried out 24 hours later

Peridural anaesthesia was caused by injecting 0 1 ml of the anaesthetic solution through a plastic catheter with the tip in the peridural space at L 2 Data on motor blockade flexion reflex block using the same criteria for blocks as above and dermatome ana esthesia as tested by pin pricking on the shaven backs of the animals were obtained The different solutions were studied on separate groups of animals

For the *intracutaneous wheal test* (BÜLBRING & WAJDA 1945) 0 25 ml of the solutions was injected intracutaneously on the shaven backs of the animals Block of the skin contraction around the injected area to pin pricking with a needle within the wheal at intervals of 5 min was taken as the criterion of sensory anaesthesia

Corneal anaesthesia was studied on rabbits by the application of 0 25 ml to the con junctival sac for 0.5 min as described by WIEDLING (1952) The same animals were used for testing both enantiomers in the latter two tests but solutions of equal strength were applied as separate wheals and on different eyes respectively

For testing of *tracheo bronchial anaesthesia* 0 05 ml of the anaesthetic solutions was applied topically to the tracheo-bronchial mucosa through a plastic catheter The solu tions were considered effective if block of a mechanically provoked cough reflex oc curred

Acute intravenous and subcutaneous *toxicity* (LD50) was determined in male albino mice (18–22 g) of the NMRI strain Solutions of 0 1 and 0.5 % strength respectively, were injected (pH 6 0) Groups of 10 animals were used for each dose The method of ÅSTRÖM & PERSSON (1961) was followed for the estimation of intravenous toxicity in rabbit following repeated injections of sublethal doses of the compound. The animals received 9 consecutive injections of equal doses at 15 min intervals The toxicity was measured as the frequency and duration of loss of the righting reflex

In vitro methods

Sciatic nerves from *Rana pipiens* were dissected cleaned from accompanying fascia and cut free of branches of the nerve a few milimeters from their bifurcation points except for the peroneal branch De sheathing the nerve was performed under a dissecting microscope starting at a proximal site of bifurcation The epineural sheath was grasped and drawn distally by means of fine forceps, the nerve being pulled free of the sheath along its entire length During the preparation the tissues were sprayed with Ringer s solution The nerve was mounted on a bio-electric couple consisting of a series of silver electrodes in a bar of plexi glass After attaching the proximal and distal electrodes to stimulation and recording units respectively the nerve was immersed in Ringer s solu tion and subsequently checked for evoked action potential Any nerve that did not respond with a normal and stable spike (A) was excluded from further trials

For the rate block experiments a portion (1 5 cm) of the nerve was soaked in the test solution (pH 7 4) in a 4 ml bath The stimulus (0 55 msec pulses at frequencies of 30 pulses/sec 2 5 V) was delivered by a Grass stimulator (S4E) and a stimulus isolation unit Recordings of the fall of the amplitude of the compound A spike were made against time from the display of the evoked action potential on a Textronic oscillo scope (502) The recovery from block was followed on washing (constant rate, about 16 7 ml/min) the nerve with Ringer s solution free of the compound The enantiomers were compared on the same nerves testing each concentration on four nerves and changing the sequence of the experiments

In the series of equilibrium blocks stimulation and recording were done in the same manner as above However in this case the whole nerve was exposed to a large volume (25 ml) of the test solution After lifting the nerve from the bath recordings of the amplitude of the action potential were made for one min at 5 min intervals This procedure was repeated until a stable state of block was attained Three identical read ings were taken as the criterion for equilibrium

Statistics

The method of MILLER & TAINTER (1944–45) was followed for the calculation of the LD50 values Significance of difference was calculated according to Student s t test Other values of statistical significance were determined according to Wilcoxon rank test (SNEDECOR & COCHRAN 1967) P values $\leq$ 0 05 were considered indications of sta tistically significant differences

Results

A Local anaesthetic effect

Comparison of R(+)-HS 37 and S(–) HS 37 was made by a number of techniques for the test of local anaesthesia following injection, infiltration and on topical application

Major nerve blocks Varying concentrations of the enantiomers in a volume of 0 2 ml were injected for (1) sciatic nerve block and (2) block of the plexus brachialis The solutions contained adrenaline 1 200,000 Using weak solutions of equal concentrations, the number of successful sciatic nerve blocks was greater with R(+)-HS 37 than with S(–) HS 37 (table 1A) Increasing the concentrations both enantiomers produced a high incidence of block The results indicate a linear relationship between duration and the log concentration (dose) with both compounds With few exceptions the R(+) form caused a significantly longer duration of loss of the motor function and flexion reflex than the S(–)-form over the concentration range

Table 1

Sciatic nerve block (A) and block of the brachial plexus (B) in the guinea pig Injection of 0.2 ml of solutions with adrenaline 1 200 000 Each solution was tested on groups of 6–12 (A) and 8–14 (B) animals respectively Figures in brackets indicate the frequency of block in per cent Unless indicated, block was caused in all animals

Compound	Duration ± S E M, min				
	0 0625 %	0 125 %	0.25 %	0.5 %	1 0 %
A *Sciatic nerve block*					
1 Motor paralysis					
S(–)-HS 37	49 ± 7(88)	87 ± 8	148 ± 10	149 ± 10	205 ± 20
R(+) HS 37	95 ± 6	130 ± 7	166 ± 10	224 ± 17	276 ± 13
Significance of difference P	< 0 01	< 0 01	N S	< 0 05	< 0 05
2 Flexion reflex block					
S(–) HS 37	29 ± 8(50)	64 ± 10(83)	95 ± 9	128 ± 6	151 ± 12
R(+)-HS 37	72 ± 9	89 ± 10	137 ± 6	163 ± 9	215 ± 7
Significance of difference P	< 0 05	N S	< 0 01	< 0 05	< 0 01
B *Plexus brachialis block*					
1 Motor paralysis					
S(–) HS 37		47 ± 8(60)	59 ± 10(75)	68 ± 9(88)	94 ± 9
R(+)-HS 37		67 ± 3	111 ± 4	130 ± 6	145 ± 4
Significance of difference, P		< 0 05	< 0 01	< 0 01	< 0 01

investigated The duration of motor paralysis exceeded that of flexion reflex loss by approximately 30 per cent irrespective of concentration and compound used

Similar results were obtained in brachial plexus blocks (table 1B) Thus treatment with R(+)-HS 37 resulted in fewer failures and blocks of a longer duration as compared to S(–)-HS 37 It follows from table 1 that the dose of the S(–)-form had to be two to four times higher than that of the R(+)-form in order to produce a block of the same duration Comparison of the sciatic nerve block and the plexus brachialis block data indicates that with the same doses the incidence of block was higher and

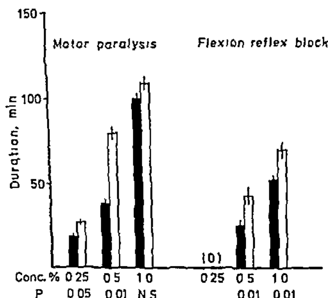


Fig 1 Peridural anaesthesia in the guinea pig Duration ± S E M of motor paralysis and flexion reflex block with S(–) HS 37 (black bars) and R(+)-HS 37 (open bars) 0 1 ml of solutions without vasoconstrictor injected at L 2 Frequencies of block and mean onset times with the various solutions are shown in the table below

Conc per cent	Motor paralysis				Flexion reflex block				n	
	Frequency per cent		Onset min		Frequency per cent		Onset min			
	S(–)	R(+)	S(–)	R(+)	S(–)	R(+)	S(–)	R(+)	S(–)	R(+)
0 25	25	86	2 0	1 2	0	0	–	–	8	14
0 5	94	100	0 6	< 0.5	62	75	5 5	4 7	16	16
1 0	100	100	< 0 5	< 0 5	100	100	4 7	2 8	14	[illegible]

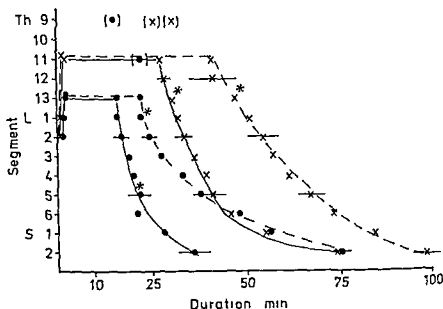


Fig 2 Peridural anaesthesia in the guinea pig Segmental spread and duration of skin anaesthesia to pin pricking 0 1 ml of 0 5 % solutions was injected at L 2 The curves indicating the decrease of the effect are drawn from the level of anaesthesia obtained in at least 50 per cent of the animals The highest level of anaesthesia is shown by symbols in brackets The sign '* denotes the highest dermatome blocked in all animals ●—● = S(–)HS 37 (n=8) ● ● = S(–) HS 37 with adrenaline 1 200 000 (n=10) ×—× = R(+)-HS 37 (n=8) and × × = R(+) HS 37 with adrenaline 1 200 000 (n=9) Duration ± S E M for some of the segments is indicated by horizontal lines The differences between S(–) HS 37 and R(+) HS 37 were statistically significant (P ≤ 0 05) for both control solutions and solutions with adrenaline

the duration of action (motor paralysis) was longer in the sciatic nerve blocks This may be related to a less rapid absorption of the drug on application to the space surrounding the sciatic nerve as compared to that around the brachial plexus With regard to the time of onset, the block occurred rapidly in both tests With the exception of the weakest solutions, motor paralysis and flexion reflex block were produced by both compounds within one and two min respectively (means) Since anaesthesia set in so quickly, it was not technically possible to demonstrate any minor difference between the enantiomers as regards the time of onset of block

(3) Peridural anaesthesia In a first series, the enantiomers were studied at three dose levels by the injection of 0 1 ml of 0 25, 0 5 and 1 0 % solutions without any vasoconstrictor The animals were checked after injection for loss of weight support in the hind legs, loss of the flexion reflex and for dermatome anaesthesia The results are shown in fig 1 A 1 0 % solution of both R(+)- and S(–)-HS 37 produced excellent frequency of motor block-

ade Failures were recorded with the two lower concentrations, but the number of successful blocks with the R(+)-form was higher The incidence of flexion reflex block with the 1 0% solution was excellent while the 0.5% solutions produced only partial blocks and 0 25% solutions completely failed to block the flexion reflex Fig 1 also shows that R(+)-HS 37 caused a longer duration of both motor paralysis and flexion reflex block with the exception of motor paralysis after the injection of 1 0% solutions The R(+)-enantiomer tended to have a slightly shorter onset time of block than the S(–) form

A higher potency of R(+) HS 37 was also found in the test for the seg-

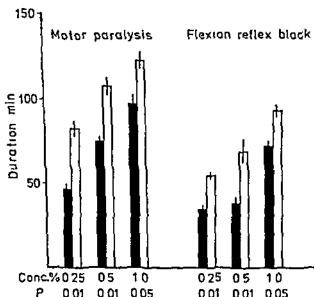


Fig 3 Peridural anaesthesia in the guinea pig Duration ± S E M of motor paralysis and flexion reflex block with solutions of S(–)-HS 37 (black bars) and R(+) HS 37 (open bars) containing adrenaline 1 200 000 Injected volume 0 1 ml The frequencies and mean onset times of block for the solutions are seen in the table below For comparison with results with solutions free of vasoconstrictor, see fig 1

Conc per cent	Motor paralysis				Flexion reflex block				n	
	Frequency per cent		Onset time		Frequency per cent		Onset time			
	S( )	R(+)	S(–)	R(+)	S(–)	R(+)	S(–)	R(+)	S(–)	R(+)
0 25	75	92	1 0	0 6	67	70	5 6	6 2	12	12
0 5	85	100	1 8	0.5	67	94	5 8	4 6	20	18
1 0	100	100	0.5	0.5	88	100	5 8	3 2	16	16

mental spread and duration of the skin anaesthesia to pin-pricking On injection of 0 25 % solutions, the R(+)-form caused block of the skin reflex of short duration and the effect did not spread above the level of the injection (L2) With the same concentration of the S(–)-form no dermatome anaesthesia could be recorded with certainty In 0 5 % solution R(+)-HS 37 reached the level of Th 12–13 in more than 90 % of the animals as compared to 50 % with S(–)-HS 37. As seen from the composite segment/time diagram in fig 2, R(+)-HS 37 also caused a greater duration of the dermatome anaesthesia than S(–)-HS 37.

The addition of adrenaline (1 200,000) to the solutions improved the effects of the compounds to a certain extent (fig 3) With the two lower concentrations (0 25 and 0 5 %) the frequencies and durations of the different types of block increased After the injection of 1 0 % solutions the duration of motor paralysis, contrary to that of flexion reflex block, was not significantly influenced by the presence of adrenaline Adrenaline did not shorten the times of onset of block In agreement with the previous series of experiments, when solutions free of vasoconstrictor were used, R(+)-HS 37 consistently displayed a higher potency than S(–) HS 37 with regard to both duration of block of weight support and duration of loss of flexion reflex A significantly better effect of the R(+)-enantiomer was also seen concerning the spread and the duration of the segmental anaesthesia (fig 2) Adrenaline enhanced the effect of both compounds

Infiltration anaesthesia A comparison of intradermal potency was made in a (4) wheal test by the injection of 0 25 ml of different concentrations (0 01–0 5 %, n–4) of the compounds The racemate was included in this trial Extrapolation from log dose-response curves showed that the concentration of R(+)-HS 37 which produced 50 per cent anaesthesia to pin-pricking was 0 06 % and 0 09 % and 0 17 % for the racemate and S(–)-HS 37 respectively Thus, the potency was in increasing order S(–) form, racemate and R(+) form

Topical anaesthesia The R(+)-enantiomer of HS 37 had the lowest threshold anaesthetic concentration, the more rapid onset and the longest duration of (5) corneal block (fig 4)

The same difference in potency between the enantiomers was seen in the second test for topical anaesthetic effect, namely (6) tracheo bronchial anaesthesia 0 05 ml of solutions of varying strength was applied to the tracheobronchial mucosa with block of the mechanically provoked cough reflex as the criterion of effectiveness Table 2 shows that R(+) HS 37 invariably caused a longer duration of action than S(–)-HS 37 With all solutions, block had already occurred at the start of testing Toxic signs were observed with a lower concentration of the R(+)-form (1 0 %) as compared to the S(–)-form (3 0 %)

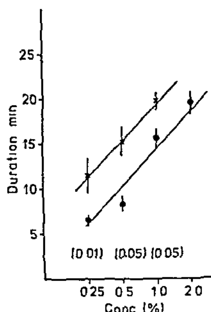


Fig 4 Corneal anaesthesia in the rabbit Duration ± S E.M of block with R(+) HS 37 (x) and S(−) HS 37 (●) P values are shown in brackets 0 25 ml of the solution was applied for 0 5 min n = 6 The frequencies of block and the mean onset times with the different concentrations are shown in the table below

Conc per cent	Frequency, per cent		Onset min		P
	R(+)	S(−)	R(+)	S(−)	
0 25	100	33	0 7 ± 0 1	3 0 ± 0 5	< 0 01
0 5	100	100	0 4 ± 0 1	1 0 ± 0 2	< 0 05
1 0	100	100	0 3 ± 0 2	1 2 ± 0 2	< 0 01
2 0	–	100	–	0 6 ± 0 1	

B Block of evoked action potential in vitro

In a first trial the decrease in the evoked action potential was determined against time following exposure of a portion of isolated sciatic nerve, with intact epineural sheath, to varying concentrations of the enantiomers With R(+)-HS 37 complete block was obtained at a lower concentration, about 0 2 mM, as compared to about 0 5 mM with the S(−)-form The results of an experiment in which nerves were immersed in a concentration (2 5 mM) of the enantiomers much higher than the approximate threshold anaesthetic concentrations for a fixed period of time (20 min) and then followed by wash out are illustrated in fig 5 It follows that the R(+)-enantiomer produced both a greater depth of block and block of a longer duration than the S(−)-

Table 2

Tracheo-bronchial anaesthesia in the guinea pig Block of mechanically induced cough reflex after topical application of 0.05 ml of the solutions to the tracheo bronchial mucosa The number of animals used for each solution is shown in brackets

Compound	Duration, ± S.E.M., min				
	0.5 %	1.0 %	1.5 %	2.0 %	3.0 %
S(–)-HS 37	5.8 ± 0.3 (6)	8.9 ± 0.2 (7)	14.6 ± 0.9 (6)	25.9 ± 0.8 (7)	33.5 ± 2.9 (6)
R(+) HS 37	16.4 ± 3.5 (4)	21.3 ± 3.0 (6)	40.1 ± 1.2 (3)	46.0 ± 2.5 (3)	
Significance of difference, P	< 0.01	< 0.01	< 0.01	< 0.01	

enantiomer In this case normal conduction with the S(–) form was seen about 200 min after the start of washing as compared to about 400 min for the R(+) form

In a second series of experiments, weak solutions were used for equilibrium blocks on both intact and de-sheathed nerves Fig 6 shows a typical experiment from a run on de-sheathed nerves The fall in the amplitude of the action potential at a steady state was clearly greater with the R(+) enantiomer (87 ± 5 %) than with the S(–) form (45 ± 2 %) The effect of the racemate in a separate test was between that of the enantiomers (65 ± 7 % block)

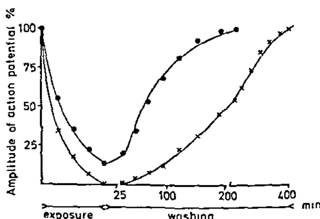


Fig 5 The effect of 2.5 mM (pH 7.4) S(–) HS 37 (●) and R(+)-HS 37 (x) on evoked action potential in isolated sciatic nerve of the frog Exposure for 20 min followed by wash-out with solution free of compound The symbols show the mean fall in the amplitude of the action potential from experiments on 4 nerves The enantiomers were tested on the same nerves with alternating sequence

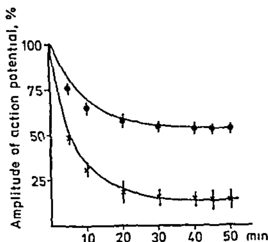


Fig 6 Equilibrium blocks of evoked action potential in isolated sheathed sciatic nerve of the frog Mean fall ± S E M in the amplitude of the action potential on exposure to 62 5 μM S(−) HS 37 (●) and R(+)-HS 37 (x) Each solution (pH 7 4) was tested on 4 different but paired nerves

The time to equilibrium for the compounds was the same The log concentration–response plot (fig 7) from the tests on de-sheathed nerves shows a ratio of activity between R(+)-HS 37 and S(–)-HS 37 of almost 3 to 1 (EC50 with R(+)-HS 37 about 4 5 μM as compared to approximately 12 μM with S(–) HS 37)

The pH dependent of the block was tested on sheathed nerves With both

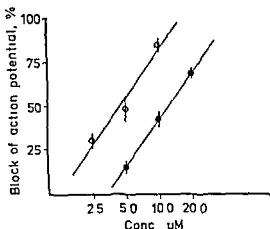


Fig 7 Equilibrium blocks in de-sheathed sciatic nerves of the frog varying concentrations (pH 7 4) of S(−)-HS 37 (●) and R(+) HS 37 (○) Each point is the mean ± S E M of 4 trials on different but paired nerves

Table 3

pH dependence of equilibrium blocks with the enantiomers of HS 37 on sheathed sciatic nerves of the frog The solutions (0 05 mM) were tested on 8 nerves at each pH

Compound	Block of AP ± S E M, per cent			
	pH			
	5 0	6 0	7 0	8 0
S(–)-HS 37	12±3	20±4	40±4	49±4
R(+)-HS 37	16±3	38±5	58±5	77±3
Significance of difference P	N S	< 0 05	< 0 05	< 0 01

compounds (pKa = 8 3) the effect increased with increase of the undissociated form in the bathing solution (table 3) With the exception of the lowest pH *the enantiomers differed significantly,* R(+) *HS 37 producing the greatest* decrease in the amplitude of the action potential

C Toxicity

Two series of experiments disclosed a higher acute toxicity for R(+) HS 37 as compared to S(–) HS 37 The intravenous LD 50 values (mice) were 8 2 ± 0 3 mg/kg for the R(+) form as compared to 20 0 ± 0 5 mg/kg for the S(–) form The corresponding values on subcutaneous administration were 49 ± 1 5 mg/kg with R(+)-HS 37 and 145 ± 4 3 mg/kg with S(–) HS 37 The results of a test in which slow repeated intravenous injections of toxic doses were given to rabbits (table 4) showed a similar difference for the enantiomers The dosage of the S(–) form had to be increased by a factor of

Table 4

Cumulative toxicity of the enantiomers of HS 37 in rabbits Mean duration ± S E M (n = 3) of loss of the righting reflex following repeated slow intravenous injections (1 0 % solutions) each of 15 min interval Experiment discontinued in case of reflex loss exceeding 15 min or death

Compound	Dose mg/kg	Duration ± S E M, min				
		Injection No				
		1	3	5	7	9
S(–)-HS 37	2 0	0	0	0	0	0
	4 0	1 7±0 9	2.7±0.9	6.5±1 0	7.2±0 7	7.8±0 4
	6 0	5 0±0 3	9.5±0.5	14 0±1.2		
R(+)-HS 37	2 0	6 7±0.3	11 7±0.3	13 0±1 0	13 7±0 7	

about three in order to produce a toxic effect equal to that obtained with the R(+) form

Discussion

This study has shown differences in the local anaesthetic potency of the enantiomers of N n butyl 3 piperidyl 2 -chloro 6 methylcarbanilate (HS 37) a new effective local anaesthetic (NILSSON *et al* 1971) R(+) HS 37 was more potent than S(–) HS 37 *in vivo* when the solutions were deposited near the free nerve ends by infiltration (wheal test) when they were applied topic ally (cornea tracheo-bronchial mucosa) and when they were injected along a composite nerve, e g sciatic nerve block and peridural anaesthesia As with other local anaesthetics the effect was improved on the addition of a vaso constrictor The R(+) form was also more active than the S(–) form on blockade of impulse conduction *in vitro* in the isolated frog nerve The potency of the racemate in infiltration anaesthesia and *in vitro* was lower than that of the R(+) form and higher than that of the S(–) form

As compared to D(–) mepivacaine L(+) mepivacaine gives a longer duration of local anaesthesia both in animal experiments (LUDUENA 1969) and in humans (ADLER *et al* 1969) Likewise (–) bupivacaine has a more prolonged effect (intracutaneous wheal test) than (+) bupivacaine (LUDUENA 1969) The (–) form of bupivacaine has the same steric configuration (S) as (+) mepivacaine (TULLAR 1971) The different durations of these pairs of enantiomers could be related to differences in effect on the surrounding vasculature (LUDUENA 1969 ABERG & WAHLSTROM 1969) Such a difference should result in variations in the systemic absorption and consequently in differences in concentration at the site of action in the nerve membrane Certain experiments suggest that concentration differences in the biophase as a result of differing rates of absorption can explain differences in the local anaesthetic effect of the enantiomers of prilocaine (AKERMAN *et al* 1967) Another possible reason for the divergent effects especially regarding duration is the differences in the rate of metabolism It has recently been shown that stereo selectivity in the enzymatic biotransformation of the enantiomers of prilocaine can occur (AKERMAN & ROSS 1970)

The results with the HS 37 enantiomers differ in some important respects from those with the pairs mentioned above R(+) and S(–) HS 37 in a number of tests showed different threshold concentrations for block Even if it is impossible to draw conclusions on the situation in the biophase from the results *in vivo* this may be an indication of the differing activities in the axon itself This interpretation is also supported by the results of block of the evoked action potential in isolated nerve where the enantiomers of HS 37 showed clear differences in activity This is the opposite to that reported for

the enantiomers of mepivacaine (ABERG & WAHLSTROM 1969) and prilocaine (AKERMAN *et al* 1967) The potency ratios of the enantiomers *in vivo* and *in vitro* were very similar Moreover, R(+) HS 37 was more toxic than S(–) HS 37 by a quotient of the same order of magnitude as that for the nerve blocking potency The results are in good agreement with previous observations (LUDUENA *et al* 1958, AKERMAN *et al* 1966, AKERMAN *et al* 1973) that local anaesthetic activity is positively correlated to acute toxicity In this connection, it is notable that neither the prilocaine (AKERMAN & ROSS 1970) nor the mepivacaine enantiomers (LUDUENA 1969) differ in acute toxicity

The results with the HS 37 enantiomers show distinct similarities with the results of studies on a series of spirosuccinimides (AKERMAN *et al* 1967 & 1969) These enantiomers differed in their nerve blocking effect both *in vivo* and *in vitro* and had dissimilar toxicities The findings on isolated excitable tissue were of special interest, significant differences in block also being obtained in preparations such as crayfish ganglia, guinea pig atria and single nerve fibres and muscle fibres (AKERMAN & SOKOLL 1969) That the compounds attain the same concentration in the biophase seems probable The view is supported by series of experiments on isolated nerves with and without the sheath (AKERMAN 1973), which showed the same uptake of radioactive labelled enantiomers

Even if more studies are needed to clarify this point, the results obtained so far with the HS 37 enantiomers as well as with the enantiomers of the spirosuccinimides (AKERMAN *et al* 1967 & 1969) and aminoacylephedrines (SCHÖNENBERGER *et al* 1967) suggest the existence of stereo-selectivity in the action of local anaesthetics in nerve impulse propagation However, the relatively small activity ratios obtained in this study, as well as those quoted indicate that comparatively little stereo sensitivity is involved in the mechanism of action of local anaesthetics, in relation to other actions of drugs on excitable tissue (PORTOGHESE 1970)

REFERENCES

Aberg G & B Wahlstrom Vascular effects of mepivacaine *Acta Physiol Scand* 1969 Suppl 330 71

Adler R G Adler & G Aberg Effects of optically active isomers and racemate of mepivacaine (carbocaine®) in dental anaesthesia *Svensk Tandläkare Tidskrift* 1969 62 501–504

Akerman B Uptake and retention of the enantiomers of a local anaesthetic in isolated nerve in relation to different degrees of blocking nervous conduction *Acta pharmacol et toxicol* 1973 32 in press

Akerman B., A Åstrom S Ross & A Telc Studies on the absorption distribution and metabolism of labelled prilocaine and lidocaine in some animal species *Acta pharmacol et toxicol* 1966 24 389–403

Akerman, B, G Camougis & R Sandberg Studies with optical antipodes of compounds possessing local anaesthetic activity *Acta pharmacol et toxicol* 1967, 25, suppl 4, 52

Akerman, B, G Camougis & R Sandberg Stereoisomerism and differential activity in excitation block by local anaesthetics *Eur J Pharmacol* 1969, 8, 337–347.

Akerman, B, N H Persson & C Tegnér Local anaesthetic properties of the optically active isomers of prilocaine (citanest®) *Acta pharmacol et toxicol* 1967, 25, 233–241

Akerman, B & S Ross Stereospecificity of the enzymatic biotransformation of the enantiomers of prilocaine (citanest®) *Acta pharmacol et toxicol* 1970, 28, 445–453

Akerman, S. B A, S B Ross & A Telč The enzymatic hydrolysis of a series of aminoacylanilides in relation to their nerve blocking effect and toxicity *Acta pharmacol et toxicol* 1973, 32, 88–96

Akerman, B & M Sokoll The effect of a pair of enantiomers with local anaesthetic activity on action potential generation and acetylcholine sensitivity in muscle *Eur J Pharmacol* 1969, 8, 331–336

Astrom, A & N H Persson Some pharmacological properties of α methyl n propyl aminopropionanilide, a new local anaesthetic *Brit J Pharmacol Chemotherap* 1961, 16 (1), 32–44

Bulbring, E & J Wajda Biological comparison of local anaesthetics *J Pharmacol Exp Therap* 1945, 85, 78–84

Luduena, F P, J O Hoppe & J K Borland A statistical evaluation of the relationships among local anaesthetic activity, irritancy and systemic toxicity *J Pharmacol Exp Therap* 1958, 123, 269–277

Luduena, F P Duration of local anesthesia *Ann Rev Pharmacol* 1969, 9, 503–520

Miller, L. C. & M L. Tainter Estimation of the ED50 and its error by means of logarithmic probit graph paper *Proc Soc Exp Biol Med* 1944–1945, 57–58, 261–264

Nilsson, J L G, H Sievertsson R Dahlbom & B Akerman Studies on carbanilic acid esters of cyclic amino alcohols 4 Esters of pyrrolidinols and piperidinols as local anesthetics *J Med Chem* 1971, 14, 710–714

Portoghese, P S Relationship between stereostructure and pharmacological activities *Ann Rev Pharmacol* 1970, 10, 51–76

Schonenberger, H, K.-D Fuchsberger, A Petter & R Brinkmann Wechselbeziehungen zwischen Wirkstoff und Rezeptor bei lokalanasthetisch wirksamen N aminoacylephedrinen *Pharm Acta Helv* 1967, 42, 163–176

Schonenberger, H, A Petter & W Zwez Wechselbeziehungen zwischen Wirkstoff und Rezeptor bei Lokalanasthetica vom Typ der N aminoacylephedrine *Pharm Acta Helv* 1967, 42, 593–603

Shackell, L F Tests of local anesthetics by sciatic nerve block in the intact guinea pig *Anesth and Analgesia* 1935, 14, 20–22

Sievertsson H, R. Dahlbom, R Sandberg & B Akerman Studies on carbanilic acid esters of cyclic amino alcohols 5 Local anesthetic potency of the enantiomers of two N butyl 3 piperidyl carbanilates *J Med Chem* 1972, 15, 1085–1086

Snedecor, G W & W G Cochran Statistical methods Iowa State University Press, Ames, Iowa 1967

Tullar, B F Optical isomers of mepivacaine and bupivacaine *J Med Chem* 1971, 14, 891–892

Wiedling, S Contribution to the pharmacology and toxicology of xylocaine *Acta pharmacol et toxicol* 1952, 8, 117–133

Acta pharmacol et toxicol 1973, 32, 112–118

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The Inhibitory Effect of Diethylaminoethyl Diphenylvalerate (SKF 525-A) on Glucuronidation by Cultures of Rat Hepatoma Cells

By

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(Received May 24 1972, Accepted June 21 1972)

Abstract SKF 525 A at concentrations from 10^{-5} M to 2×10^{-4} M inhibits the glucuronidation of p aminophenol (PAP) p nitrophenol (PNP) and bilirubin by a clonal strain of rat hepatoma cells (MH_1C_1) grown in culture At higher concentrations of SKF 525 A the glucuronidation of PAP and PNP in a homogenate system from the cell cultures and normal rat liver is also inhibited PAP to the greatest extent The inhibition of PAP glucuronidation in the homogenate system appears to be of the non competitive type

Key words Drug metabolism – SKF 525 A – glucuronyl transferase – cell culture

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Diethylaminoethyl diphenylvalerate (SKF 525-A) is known to inhibit the glucuronidation of drugs and endogenous substrates COOPER *et al* (1954) noted a decreased conjugation of morphine in rabbit and rat liver slices after the addition of SKF 525-A The glucuronidation of *o* aminophenol and *p* nitrophenol in rat and guinea pig homogenates and of bilirubin in rat liver slices and homogenates was inhibited by SKF 525-A (HARGREAVES 1967) IKEDA *et al* (1968) have shown an *in vivo* effect of the inhibitor on phenolic glucuronidation in rats and guinea pigs They also found that the inhibition of *o* aminophenol glucuronidation by guinea pig liver homogenates was of the non competitive type SKF 525 A blocked the glucuronidation of *p*-nitrophenol in intact isolated rat hepatocytes, but not in a homogenate from the cells (HENDERSON & DEWAIDE 1969), thus suggesting a change at the level of the cell membrane On the other hand, the inhibition of ^{3}H-tetrahydrocortisone glucuronidation in rat liver microsomes by SKF 525-A appeared to be competitive in nature (MILLER & DINGELL 1971)

We have used a clonal strain of rat hepatoma cells that glucuronidates bilirubin (RUGSTAD *et al* 1970), *p* aminophenol (DYBING & RUGSTAD 1972) and *p* nitrophenol (DYBING 1972) to investigate the possible inhibitory

effects of SKF 525-A in this system. The use of a clonal cell line with several liver specific functions intact makes it possible to study performances of living cells under strictly controlled conditions Differences in effects in whole cells as compared to the effects seen in homogenates could point to sites of action of the inhibitor

Materials and Methods

Methods of cell culture

The clonal strain MH_1C_1 of rat hepatoma cells was grown in Dulbecco s modified Eagle medium supplemented with 15 % horse serum and 2 5 % foetal calf serum and antibiotics as previously described (DYBING & RUGSTAD 1972)

Cell culture experiments

Replicate, fullgrown subcultures (12–15) were incubated with substrates in fresh serum-containing medium without or with inhibitor in a total volume of 10 ml for 4 hours at 37° The appearance of p aminophenyl (PAP) glucuronide or bilirubin glucuronide or disappearance of p-nitrophenol (PNP) was assayed in aliquots of the incubation mediums Substrate concentrations were for PAP and PNP 0 20 mM and for bilirubin 0 10 mM Bilirubin was dissolved in a small volume of NaOH diluted to make an equimolar solution of human albumin In the bilirubin experiment no protein other than the albumin was present in the medium

Homogenate experiments

The rat hepatoma cells in culture were removed with an icecold 0 02 % EDTA barbital/NaCl buffer pH 7 4 spun down at 2000 r p m and homogenized for 10 minutes in a glass homogenizer with a teflon pestle in a 125 mM tris maleate buffer pH 7 4 pestle speed 1000 r p m The homogenate was centrifugated at 700 × g for 5 minutes at 4° and the supernatant was used as source of enzyme In the rat liver experiments livers from male Wistar rats were removed chilled and cut up with scissors Four g of liver was homogenized in 5 ml 125 mH tris maleate pH 7 4 with 5 strokes of a teflon glass homogenizer The homogenate was centrifugated at 2000 × g for 15 minutes the supernate was diluted to make a 45 mg/ml protein solution The incubation mixtures contained 0 1 ml of one of the types homogenate the final concentrations of PAP or PNP being 0 20 mM (unless otherwise stated) UDPGA 4 0 mM tris maleate 75 mM pH 7 4 $MgCl_2$ 10 0 mM and SKF 525 A at various concentrations in a total volume of 0 5 ml Incubations were carried out in a water shaking bath at 37° For PAP the incubation time was 30 minutes both in homogenates of MH_1C_1 cells and rat liver For PNP 5 minutes incubation time was used for MH_1C_1 cell homogenates and 20 minutes for rat liver homogenates Reactions were stopped with 0 5 ml ice cold 0 5 M TCA Cell protein was determined both in cell culture and homogenate experiments according to OYAMA & EAGLE (1956) using bovine serum albumin as standard.

Drug assays

The following assays were used PAP glucuronide formation was determined according to DYBING & RUGSTAD (1972) PNP disappearance as described by ISSELBACHER *et al* (1962) and bilirubin glucuronide formation by the method of WEBER & SCHALM (1962)

Table 1

The effects of SKF 525 A on glucuronidation of PAP PNP and bilirubin in cultures of MH_1C_1 cells PAP 0 20 mM and PNP 0 20 mM in medium containing 17 5 % serum or bilirubin 0 10 mM in medium with 0 10 mM albumin were incubated for 4 hours at 37° without or with 0 10 mM and 0 20 mM SKF 525 A respectively Values are means ± S D , numbers of flasks in brackets

Substrate	Substrate alone nmoles/mg prot × hrs	With SKF 525 A 0 10 mM nmoles/mg prot × hrs	With SKF 525 A 0 20 mM nmoles/mg prot × hrs
p Aminophenol	51 72 ± 2 41 (4)	33 05 ± 1 08 (4)	19.36 ± 5 15 (4)
p Nitrophenol	92 41 ± 3 89 (4)	50 58 ± 5 79 (4)	31 36 ± 10 21 (4)
Bilirubin	21 63 ± 8 23 (4)	4 40 ± 3 04 (4)	–

Chemicals

Para aminophenol (PAP) and *p* nitrophenol (PNP) were obtained through Norsk Medisinaldepot (NMD) 2 Diethylaminoethyl 2 2 diphenylvalerate HCl (SKF 525 A) was a gift from the Smith Kline and French Laboratories UDPGA (triammonium salt) and bilirubin was purchased from the Sigma Company human albumin from Kabi

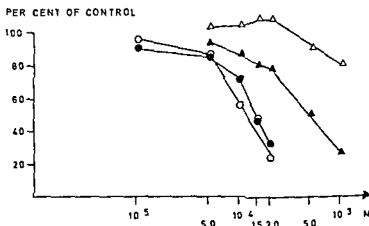


Fig 1 Semi logarithmic plot of inhibition of PAP and PNP glucuronidation in cell cultures and homogenates by SKF 525 A 0 20 mM of substrates were incubated in MH_1C_1 cell cultures for 4 hours at 37° Values in per cent of controls (3 parallels not shown in the figure) from duplicate flasks for each concentration of SKF 525 A Also shown is the effect of various concentrations of SKF 525 A on the glucuronidation *of 0 20 mM PAP and PNP in a homogenate system from* the MH_1C_1 cells (3 estimations for each concentration of SKF 525 A controls not shown) ●——● PAP in cell culture ▲——▲ PAP in cell homogenate ○——○ PNP in cell culture △——△ PNP in cell homogenate

Table 2

Effect of various concentrations of SKF 525 A on glucuronidation of PAP and PNP in homogenates from rat hepatoma cells (MH_1C_1) and normal rat liver Activity in nmoles per mg protein and minute Values are means of three parallel estimations

Incubation system	Substrate	Control	With addition of SKF 525 A 0 05 mM	0 10 mM	0 15 mM	0.20 mM	0.50 mM	1 0 mM
MH_1C_1 cell culture homogenate	PAP	9 64	8 89	8 33	7 64	7 41	4 77	2 48
	PNP	63 60	65 53	66 25	68 65	68 29	56 97	50 39
Rat liver homogenate	PAP	0 32	0.33	0.32	0 28	0 27	0 20	0 22
	PNP	0 73	0 72	0 73	0 69	0 65	0 62	0 71

Results

Table 1 shows that SKF 525 A at concentrations of 0 10 and 0 20 mM diminishes the rate of glucuronidation of three different substrates in the cell cultures At 0 20 mM SKF 525-A cellular detachment was noted in the experiment with bilirubin, this phenomenon being first apparent at 0 25 mM SKF 525 A and above in the PAP and PNP experiments Albumin concentration in the mediums was approximately equal using 17 5 % serum or 0 10 mM albumin

A log dose response curve (LDR) of SKF 525-A effects on the glucuronidation of PAP and PNP in the cell culture system is seen in fig 1 The two LDR-curves are fairly similar, inhibition being detectable from 10^{-5}M and 50 % inhibition at $1\ 25 \times 10^{-4}$M

Fig 1 also gives a LDR plot of SKF 525-A on the glucuronidation of PAP and PNP by UDP-glucuronyl transferase in a homogenate system from the MH_1C_1 cells Both reactions are inhibited at much higher concentrations of SKF 525-A than in the cell cultures, but to different extents PAP glucuronidation is affected to the greatest degree, with 50 % inhibition at 5×10^{-4}M, PNP glucuronidation in the homogenate system much less so, giving 80 % of control values at 10^{-3}M

Table 2 gives the values of PAP and PNP glucuronidation at various SKF 525-A concentrations in a homogenate prepared from the MH_1C_1 cell cultures as compared with those seen in a normal rat liver homogenate It is apparent that the velocities in the cell culture homogenate is 30–90 times higher SKF 525-A inhibits the glucuronidation of both substrates in the normal rat liver homogenate, but to a somewhat less percentual extent as compared to homogenates from MH_1C_1 cells

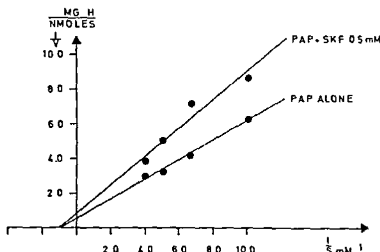


Fig 2 Double reciprocal plot of PAP glucuronidation in MH_1C_1 cell culture homogenate without or in the presence of 0 5 mM SKF 525 A Substrate concentrations were 0 10 0 15, 0.20 and 0 25 mM respectively

From fig. 2 it appears that the SKF 525 A inhibition of PAP glucuronidation in the cell culture homogenate is of the non-competitive type, the apparent K_m here being 1 0 mM A corresponding experiment with PNP was not carried out, the inhibition after SKF 525-A using this substrate not being sufficiently striking in the concentration range used

Discussion

As in other systems, SKF 525-A inhibits glucuronidation of exogenous and endogenous substrates in cultures of rat hepatoma cells At concentrations above 2 0 × 10^{-4}M, the cells show signs of toxic damage and eventually become detached Using bilirubin this effect was seen with a dose as low as 0 20 mM SKF 525 A This can be explained by the displacement by bilirubin of SKF 525-A binding to albumin thus giving higher free, active concentrations of SKF 525-A If in turn bilirubin is displaced from its binding site, lower glucuronidation values would be expected since the bilirubin albumin ratios different from 1 1 greatly diminishes bilirubin glucuronidation (BRATLID & RUGSTAD 1972) Chlorpromazine exerts greater inhibition of PAP glucuronidation by MH_1C_1 cell cultures in a serum-free as compared to a serum-containing medium (DYBING 1972)

The actions of SKF 525 A on glucuronidation are similar to those observed with chlorpromazine (DYBING 1972) At least three times the concentrations of SKF 525-A are necessary to achieve identical inhibition of PAP glucuronidation in the cell homogenate as compared to the cell cultures

The homogenate incubation media contain no other protein than the enzyme source

The addition of serum also diminished the inhibitory effect of chlorpromazine on PAP glucuronidation in MH_1C_1 cell homogenates (DYBING 1972) The effect on PNP glucuronidation by SKF 525 A in the cell cultures is probably due to effects other than that on the enzyme (glucuronyl transferase) These findings might suggests that SKF 525 A exerts part of its actions before enzymatic coupling, as HENDERSON & DEWAIDE (1969) have suggested i e presumably a membrane effect

It is interesting to note the different velocities of glucuronidation in the cell homogenate as compared to those seen with normal rat liver The enzyme from the rat hepatoma cells appears to be fully induced and almost fully activated (WINSNES & RUGSTAD, unpublished results) In the normal rat liver homogenate, SKF 525-A has inhibitory actions on the glucuronidation of PAP and PNP also

SKF 525-A as well as chlorpromazine has profound inhibitory effects on alanine incorporation into protein by these liver cells in culture (DYBING, unpublished results) Part of these actions can be explained by inhibition of amino acid uptake, as shown by experiments using the nonmetabolizable amino acid α-aminoisobutyric acid BRODIE (1956) suggested that SKF 525-A might interact with the microsomal membrane, thereby decreasing its permeability to drugs SKF 525-A stabilizes erythrocytes against hypotonic haemolysis (LEE *et al* 1968) and this stabilization appears to correlate well with the degree of surface activity, which is comparable to that of chlorpromazine

The greater effect on glucuronidation of two different substrates in intact, living cells as compared to homogenates suggests that SKF 525-A inhibits the entrance of these substrates into the cells

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REFERENCES

Bratlid D & H E Rugstad The effect of varying the molar bilirubin/albumin ratio on bilirubin conjugation and toxicity in cell culture of a clonal strain of rat hepatoma cells *Scand J Lab Clin Invest* 1972 29 461–465

Brodie B B Pathways of drug metabolism *J Pharm Pharmacol* 1956 8 1–17

Cooper, J R, J Axelrod & B B Brodie Inhibitory effects of β diethylaminoethyl diphenylpropylacetate on a variety of drug metabolic pathways *in vitro* *J Pharmacol Exp Therap* 1954, **112**, 55–63

Dybing, E Chlorpromazine inhibition of *p* aminophenol glucuronidation by rat hepatoma cells in culture *Acta pharmacol et toxicol* 1972, **31**, 287–295

Dybing, E & H E Rugstad *Para* aminophenol metabolism by a clonal strain of rat hepatoma cells with liver like functions *Acta pharmacol et toxicol* 1972, **31**, 153–160

Hargreaves, T The effect of diethylaminoethyl diphenylpropyl acetic acid (SKF 525 A) on uridine 5' pyrophosphate glucuronyltransferase *Biochem Pharmacol* 1967, **16**, 1481–1488

Henderson, P T & J H Dewaide Metabolism of drugs in isolated rat hepatocytes *Biochem Pharmacol* 1969, **18**, 2087–2094

Ikeda, M, S Tanaka & T Katayama Inhibitory effect of 2 diethylaminoethyl 2 2 diphenylvalerate hydrochloride (SKF 525 A) on aromatic hydroxylation and phenolic glucuronidation *Mol Pharmacol* 1968, **4** 38–43

Isselbacher, K J, M Chrabas & R C Quinn The solubilization and partial purification of a glucuronyl transferase from rabbit liver microsomes *J Biol Chem* 1962, **237**, 3033–3036

Lee, I P, H I Yamamura & R L Dixon The effects of β diethylaminoethyl diphenylpropylacetate (SKF 525 A) on biological membranes – I SKF 525 A induced stabilization of human erythrocytes *Biochem Pharmacol* 1968, **17**, 1671–1681

Miller, K W & J V Dingell The interaction of drugs with the conjugation of tetrahydrocortisone and a method for the measurement of the formation of its glucuronide, *in vitro* *J Pharmacol Exp Therap* 1971, **178**, 602–608

Oyama, V I & H Eagle Measurement of cell growth in tissue culture with a phenol reagent (Folin Ciocalteau) *Proc Soc Exp Biol Med* 1956, **150**, 305–308

Rugstad H E, S H Robinson, C Yannoni & A H Tashjian jr Metabolism of bilirubin by a clonal strain of rat hepatoma cells *J Cell Biol* 1970, **47** 703–710

Weber, A P H & L Schalm Quantitative separation and determination of bilirubin and conjugated bilirubin in human serum *Clin Chim Acta* 1962, **7**, 805–809

Acta pharmacol et toxicol 1973, 32, 119–128

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Modification by Methyltyrosine Methylester (H 44/68) of the Amphetamine-Induced Toxicity and Brain Catecholamine Changes in Developing Mice

By

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Abstract The effect of methyltyrosine methylester (H 44/68) on the brain levels of noradrenaline (NA) and dopamine (DA) 16 hours after the administration in adult and in developing mice aged 3–5 days and 13–15 days was studied In the youngest mice 250 mg/kg of H 44/68 markedly decreased the brain catechol amines (CA) whereas in 13–15-day old mice even 500 mg/kg of H 44/68 failed to reduce the DA level After this dose NA was decreased to 25 % In adult brains both doses of H 44/68 caused pronounced reduction of the CA levels, DA decreasing more than NA High doses of amphetamine (50–120 mg/kg) given to H 44/68 pretreated mice were less toxic than without pre treatment Amphetamine generally enhanced the H 44/68 induced depletion of brain CA, except in the youngest mice in which amphetamine (120 mg/kg) counteracted the brain DA depletion induced by H 44/68 It is suggested that the varying changes in the CA levels in H 44/68 treated developing mice might result from differences in the turnover rates of CA during development Enhancement by amphetamine of H 44/68 induced reduction in CA reflects the accelerating effect of amphetamine on CA metabolism

Key words Age – amphetamine – catecholamines – methyltyrosine

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A relative tolerance to amphetamine toxicity previously demonstrated in developing mice (ALHAVA 1972) could not be adequately explained by the amphetamine induced changes in the brain levels of catecholamines (CA) in different age groups (ALHAVA & KLINGE 1972) There was lack of direct correlation between the brain CA depletion and amphetamine toxicity, which was assumed to reflect complex mechanisms of action of the drug on central CA neurons (ALHAVA & KLINGE 1972)

It is generally accepted that the central action of amphetamine depends on an undisturbed CA biosynthesis (WEISSMAN *et al* 1966, HANSON 1967) Inhibition of tyrosine hydroxylase by methyltyrosine provides a complete pro-

tection against amphetamine toxicity in mice (MENON & DANDIYA 1967) Furthermore, the typical amphetamine syndrome induced by small doses of the drug is not seen after inhibition of tyrosine hydroxylase and the stimulating effect is restored upon administration of L-DOPA (RANDRUP & MUNKVAD 1966, HANSON 1967) In developing mice, the primarily low central CA level was assumed to provide at least partial protection against amphetamine toxicity (ALHAVA & KLINGE 1972) Therefore, it seemed to be of interest to study the effect of an inhibition of the CA biosynthesis on the amphetamine induced toxicity and changes in the central CA levels also in developing mice

Material and Methods

Adult male NMRI strain mice weighing 22 8 ± 0.5 g (mean ± S E M) and developing mice of either sex in the age groups of 3–5 days and 13–15 days were used The body weights of developing mice were 2 68 ± 0 09 g and 5 98 ± 0 11 g respectively The adult mice were given standard laboratory pellets (Hankkija Oy, Helsinki) and water *ad libitum* and housed in their home cages (20×35×13 cm 20–30 mice in each) until used The developing mice were kept with their mothers until the time of the experiments

The mice were injected intraperitoneally, at 4 p m with methyltyrosine methylester (H 44/68, Kistner Lab., Gothenburg Sweden) in saline solution in a single dose of 250 mg/kg or in two subsequent doses of 250 mg/kg at 1 hour interval The injection volume was constantly 0 1 ml/10 g of body weight The injection technique used for the animals weighing less than 10 g has been previously described (ALHAVA 1972) After the injection the adult mice were housed in groups of 4 animals and given food and water *ad libitum* 16 hours after the latter injection Developing mice treated with methyltyrosine methylester were returned to their mothers for 16 hours after which time the CA content of the whole brains was assayed

Mice pretreated with H 44/68 16 hours previously were injected intraperitoneally with toxic doses (50–120 mg/kg) of dl amphetamine sulphate dissolved in distilled water After the amphetamine injection the mice were put into opaque plastic cages (15×20× 13 cm) in groups of 4 without access to food or water They were decapitated im mediately after spontaneous death or after the observation period of 4 hours The whole brains were assayed for their CA content

The technique used for the determination of noradrenaline (NA) and dopamine (DA) was a slight modification of the spectrophotofluorometric methods of BERTLER *et al* (1958) and CARLSSON & WALDECK (1958) as previously described (ALHAVA & KLINGE 1972) The adult brains were analyzed individually In developing mice each sample consisted of 4 pooled brains In 13 determinations the recovery of NA was 61 1 ± 2.5 % and that of DA 82 9 ± 2.5 % The results were corrected for the respective recoveries. When the columns were loaded with pure CA and H 44/68 the assay of CA was not interfered by H 44 68

The dose of H 44/68 refers to methylester Doses of amphetamine refer to the base The environmental temperature during the experiments with amphetamine was 24.5 ± 0.5° The brain NA and DA values are expressed in μg/g of fresh tissue Student s t test was used for the statistical treatment of the results

Table 1

Effect of H 44/68 on the brain levels of NA and DA in developing and adult mice. The animals given 250 mg/kg of H 44/68 were decapitated 16 hours after the injection. The dose of 500 mg/kg was given in two fractions of 250 mg/kg at 1 hour interval and the mice were killed 16 hours after the latter injection. The adult brains were analyzed individually. In developing mice each experiment was performed on 4 pooled brains. The number of experiments is given in brackets.

Age (days)	Dose of H 44/68 (mg/kg)	NA μg/g mean ± S.E.M.	% of control level	DA μg/g mean ± S.E.M.	% of control level
3–5	250	0.07 ± 0.01***	(5) 44	–	(5) 0
	control	0.16 ± 0.01	(6) 100	0.18 ± 0.03	(6) 100
13–15	250	0.12 ± 0.01**	(6) 60	0.25 ± 0.03	(6) 104
	500	0.05 ± 0.01***	(6) 25	0.21 ± 0.01	(5) 88
	control	0.20 ± 0.02	(6) 100	0.24 ± 0.02	(6) 100
Adult	*250*	*0.30 ± 0.01****	*(5) 70*	*0.37 ± 0.08****	*(5) 41*
	500	0.09 ± 0.02***	(5) 21	–	(5) 0
	control	0.43 ± 0.01	(10) 100	0.90 ± 0.05	(10) 100

** $P < 0.01$
*** $P < 0.001$
The significances indicate deviations from the control levels.

Results

Effect of H 44/68 on the brain CA

The effect of methyltyrosine methylester on the brain levels of NA and DA 16 hours after the administration in developing and adult mice is shown in table 1. In the youngest mice the single dose of 250 mg/kg of H 44/68 was sufficient to deplete the brain NA level to 44 % of the control level ($P < 0.001$). The brain DA level was not measureable after this treatment. In the older age group of developing mice 250 mg/kg of H 44/68 decreased the brain NA to 60 % ($P < 0.01$) whereas the DA levels remained unchanged. Two successive doses of 250 mg/kg of methyltyrosine methylester depleted the brain NA level to 25 % ($P < 0.001$). The brain DA was insignificantly decreased (to 88 %). In adult mice the effect of H 44/68 on brain DA was pronounced. The smaller dose decreased brain DA to 41 % ($P < 0.001$), while NA was depleted to only 70 % ($P < 0.001$). After 500 mg/kg of the drug the brain DA levels were not detectable by the method, and NA was also strikingly decreased (to 21 %, $P < 0.001$).

Table 2

Effect of amphetamine on the brain CA levels of 3–5 day old mice pretreated with H 44/68 16 hours earlier The mice were decapitated 4 hours after amphetamine injection The number of experiments is given in brackets

Dose of H 44/68 (mg/kg)	Dose of amphetamine (mg/kg)	Time after administration (hrs)	NA µg/g mean ± S E M	% of control level	DA µg/g mean ± S E M	% of control level
250		16	0 07 ± 0 01***	(5) 44	–	(5) 0
250	120	16 + 4	0 02 ± 0 01***	(4) 13	0 07 ± 0 03*	(4) 39
control			0 16 ± 0 01	(6) 100	0 18 ± 0 03	(6) 100

* P < 0 05
*** P < 0 001
The significances indicate deviations from the control levels

Modification by H 44/68 of the amphetamine action

Table 2 shows the effect of 120 mg/kg of amphetamine on the brain CA levels of the youngest mice pretreated with methyltyrosine methylester 16 hours previously Amphetamine enhanced the depletion of brain NA, the level of which further decreased by 31 % from values measured after H 44/68 only The total depletion of the brain DA caused by H 44/68 only was not seen after amphetamine administration In mice given 120 mg/kg of amphetamine after the pretreatment with methyltyrosine methylester the brain DA was depleted to only 39 % of the control level (P < 0 05)

In the older age group of developing mice a 100 mg/kg dose of amphetamine given 16 hours after H 44/68 caused a massive depletion of both NA and DA levels when measured 4 hours after the amphetamine injection

Table 3

Effect of amphetamine on the brain CA levels of 13–15 day old mice pretreated with H 44/68 16 hours earlier The mice were decapitated 4 hours after amphetamine injection The number of experiments is given in brackets

Dose of H 44/68 (mg/kg)	Dose of amphetamine (mg/kg)	Time after administration (hrs)	NA µg/g mean ± S E M	% of control level	DA µg/g mean ± S E M	% of control level
250		16	0 12 ± 0 01**	(6) 60	0 25 ± 0 03	(6) 104
500		16	0 05 ± 0 01***	(6) 25	0 21 ± 0 01	(5) 88
250	100	16 + 4	0 02 ± 0 01***	(7) 10	0 11 ± 0 02**	(7) 46
control			0 20 ± 0 02	(6) 100	0 24 ± 0 02	(6) 100

** P < 0 01
*** P < 0 001
The significances indicate deviations from the control levels

Table 4

Effect of amphetamine on the brain CA levels of adult mice pretreated with H 44/68 16 hours earlier. The mice were decapitated 4 hours after amphetamine injection, except for those which died within 10 minutes after 100 mg/kg of amphetamine. The number of experiments is given in brackets.

Dose of H 44/68 (mg/kg)	Dose of amphetamine (mg/kg)	Time after administration (hrs)	NA µg/g mean ± S.E.M.	% of control level	DA µg/ mean ± S.E.M.	% of control level
250		16	0.30 ± 0.01***	(5) 70	0.37 ± 0.08***	(5) 41
500		16	0.09 ± 0.02***	(5) 21	–	(5) 0
250	50	16 + 4	0.14 ± 0.01***	(6) 33	0.30 ± 0.09***	(6) 33
250	100	*16 + 0.6*	*0.23 ± 0.08***	*(2) 53*	*0.61 ± 0.04**	*(2) 68*
250	100	16 + 4	0.01 ± 0.01***	(2) 2	0.31 ± 0.01***	(2) 34
control			0.43 ± 0.01	(10) 100	0.90 ± 0.05	(10) 100

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$
The significances indicate deviations from the control levels.

(table 3). The brain NA was decreased to 10 % ($P < 0.001$) and DA to 46 % ($P < 0.01$), respectively.

Table 4 shows the effect of 50 and 100 mg/kg of amphetamine on the brain CA in adult mice pretreated with H 44/68. After 50 mg/kg of amphetamine the brain NA and DA levels were equally decreased to 33 % ($P < 0.001$). A hundred mg/kg of amphetamine was lethal to 50 % of the mice, who died within 10 minutes after the injection. In these the brain NA was decreased to 53 % ($P < 0.01$) and DA to 68 % ($P < 0.05$). The survivers were decapitated 4 hours after the amphetamine injection and the brain CA levels were found to be markedly depleted (NA to 2 %, DA to 34 %, $P < 0.001$). Because the amphetamine-treated animals were compared with animals killed only 16 hours after H 44/68, any possible changes in CA levels between 16 and 20 hours after H 44/68 are worthy of consideration although they are very small (Corrodi & Hanson 1966).

A single dose of 250 mg/kg of H 44/68 caused no behavioural effects in any of the age group studied. Sedation and fatigue were seen in adult mice injected with two subsequent doses of H 44/68. In 13–15 day old mice even the double dose of methyltyrosine methylester failed to change the gross behaviour. Pre-treatment with methyltyrosine methylester did not inhibit the motor stimulation induced by amphetamine, although the stimulation was milder than without pretreatment (Alhava 1972). The mild stimulating effect of amphetamine after pretreatment with H 44/68 was prolonged, the animals being quite active at the time of decapitation.

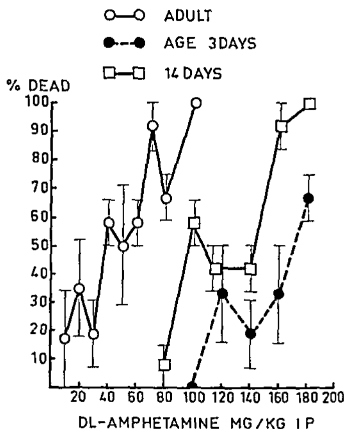


Fig 1 The dose-mortality curves for dl amphetamine in adult mice and developing mice aged 3 days and 14 days Each point of the curves represents 12 20 mice (ALHAVA 1972)

Discussion

The protective effect of methyltyrosine against amphetamine toxicity in adult mice is well documented (MENON & DANDIYA 1967), this is further supported by the present findings The pretreatment with methyltyrosine methylester offered a partial protection against 100 mg/kg of amphetamine, a dose which killed 50 % of the animals This dose was previously shown to cause a 100 % mortality rate (fig 1, ALHAVA 1972) The protective effect of the pretreatment against 50 mg/kg of amphetamine was complete when compared with the 50 % mortality rate previously found In the present study systematic toxicity experiments in methyltyrosine pretreated developing mice were not performed but during the 4 hour observation period after the administration of amphetamine no deaths were seen When comparing this with the mortality rates induced by respective doses of amphetamine previously (fig 1, ALHAVA 1972) the protection given by methyltyrosine pretreatment is also seen in developing mice Thus, the dose mortality curves for

amphetamine in methyltyrosine pretreated adult and developing mice would in all probability be shifted to the right The inhibition of the typical amphetamine syndrome after pretreatment with methyltyrosine (RANDRUP & MUNKVAD 1966) could only partially be reproduced, probably because the dose scales in these two studies were entirely different The reason for the prolonged stimulation after amphetamine in pretreated mice remains obscure and awaits further experiments on CA turnover rate

Inhibition by methyltyrosine methylester of the CA biosynthesis does not seem to be uniform in different age groups of mice In the youngest mice both the NA and DA levels were sensitive to the depleting action of the drug, while in the older age group of developing mice, a higher dose of the inhibitor was needed to reduce the brain DA content, and even so the depletion was slight In adult brains the changes in the CA levels caused by H 44/68 agree with the findings of CORRODI & HANSON (1966), the decrease of the DA level being more pronounced than that of the NA level The relative resistance of the DA pool to the inhibition of tyrosine hydroxylase seen in 13–15 day old mice suggests that in this particular age group the turnover rate of the central CA differs from that of infant and adult mice At that age the synthesis rate of DA could either be particularly high or the activity of the CA inactivating mechanisms remarkably low (MIRKIN 1970) In the rat foetus and neonate there is deficiency of both CA-degrading enzymes, the activities of which are only 20 % of that observed in the adult (GLOWINSKI *et al* 1964)

Although amphetamine enhanced the H 44/68 effects on brain CA, an exceptional phenomenon was seen in the youngest mice a high dose of amphetamine partially restored the brain DA level previously completely depleted by H 44/68 This might result from the dopamine β hydroxylase inhibiting activity of amphetamine (GLOWINSKI *et al* 1966), and, possibly, from the amphetamine induced stimulation of the biosynthesis of the brain DA demonstrated in rats (COSTA & GROPPETTI 1970) The result refers to a previous study, in which 3–5 day old mice injected with 100 mg/kg of amphetamine showed an increase in the brain DA level (ALHAVA & KLINGE 1972)

Table 5 shows the CA-depleting effect of amphetamine after *H 44/68*, as compared with the depleting effect without the pretreatment with the synthesis inhibitor (ALHAVA & KLINGE 1972) The brain NA and DA levels in animals pretreated with H 44/68 were generally lower than in animals injected with amphetamine only, although the times after the administration of amphetamine in experiments on developing mice differ An interesting exception is seen in the brain DA levels of 13–15 day old mice, in which 100 mg/kg of amphetamine reduced the brain DA level in 1 hour more than the same dose given to pretreated animals in 4 hours, although the

Table 5

Comparison of the CA-depleting effect of amphetamine after H 44/68 with the depleting effect without the pretreatment with the synthesis inhibitor in developing and adult mice (ALHAVA & KLINGE 1972) The significances indicate deviations from the control levels n = number of experiments

Age (days)	H 44/68 (mg/kg)	Amphetamine (mg/kg)	Time after amphetamine	n	NA µg/g mean ± S E M	% of control level	DA µg/g mean ± S E M	% of control level
3–5	250	120	4 hrs	4	0 02 ± 0 01***	13	0 07 ± 0 03*	39
		120	1 hr	3	0 07 ± 0 02**	44	0 18 ± 0 01	100
	control			6	0 16 ± 0 01	100	0 18 ± 0 03	100
13–15	250	100	4 hrs	7	0 02 ± 0 01***	10	0 11 ± 0 02**	46
		100	1 hr	7	0 07 ± 0 02***	35	0 07 ± 0 01***	29
	control			6	0 20 ± 0 02	100	0 24 ± 0 02	100
Adult	250	50	4 hrs	6	0 14 ± 0 01***	33	0 30 ± 0 09***	33
		50	4 hrs	8	0 15 ± 0 02***	35	0 81 ± 0 06	90
	250	100	10 min	2	0 23 ± 0 08**	53	0 61 ± 0.04*	68
		100	15–25 min.	5	0 34 ± 0 01**	79	0 64 ± 0 08*	71
	control			10	0 43 ± 0 01	100	0 90 ± 0 05	100

* P < 0 05
** P < 0 01
*** P < 0 001

difference was insignificant This might again reflect the particular type of turnover rate of central CA in this age group and, on the other hand, the dopamine β hydroxylase inhibiting activity of amphetamine (GLOWINSKI *et al* 1966)

According to AXELROD (1970), amphetamine appears to accelerate the rate of metabolism of CA in mice by preventing the protective binding in vesicles thus allowing of the free CA to be exposed to a rapid disappearance Furthermore, the drug is known to affect various processes in CA metabolism, including the uptake of CA into neurons (GLOWINSKI 1970) In the present study, the very high doses of amphetamine could also release CA from the large storage granular pool The enhancement of the H 44/68-induced depletion of brain CA by amphetamine in both adult and developing mice also reflects the accelerating effect of amphetamine on the brain CA metabolism Since the physiological importance of the newly synthesized CA for the action of amphetamine is well documented (GLOWINSKI 1970) and the turnover rates of the central CA appear to be variable at different stages of development (MIRKIN 1970) no further conclusions can be drawn from the present findings

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REFERENCES

Alhava Eeva Amphetamine toxicity in adult and developing mice *Acta pharmacol et toxicol* 1972 31 387–400

Alhava, E & E Klinge Age and brain catecholamine content as factors influencing amphetamine toxicity in mice *Acta pharmacol et toxicol* 1972 31 401–411

Axelrod J Amphetamine metabolism physiological disposition and its effects on catecholamine storage In *International symposium on amphetamines and related compounds* Ed E Costa & S Garattini Raven Press, New York, 1970 207–216

Bertler, A A Carlsson & E Rosengren A method for the fluorimetric determination of adrenaline and noradrenaline in tissues *Acta physiol scand* 1958 44, 273–292.

Carlsson A & B Waldeck A fluorimetric method for the determination of dopamine (3 hydroxytyramine) *Acta physiol scand* 1958 44 293 298

Corrodi H & L C F Hanson Central effects of an inhibitor of tyrosine hydroxylation *Psychopharmacologia (Berl)* 1966 10 116–125

Costa E & A Groppetti Biosynthesis and storage of catecholamines in tissues of rats injected with various doses of d amphetamine In *International symposium on amphetamines and related compounds* Ed E Costa & S Garattini Raven Press New York 1970 231 255

Glowinski, J, J Axelrod I J Kopin & R J Wurtman Physiological disposition of H^3-norepinephrine in the developing rat *J Pharmacol exp Therap* 1964, 146 48-53

Glowinski J, J Axelrod & L. I Iversen Regional studies of catecholamines in the rat brain IV Effects of drugs on the disposition and metabolism of H^3-norepinephrine and H^3-dopamine *J Pharmacol exp Therap* 1966, 153, 30–41

Glowinski, J Effects of amphetamine on various aspects of catecholamine metabolism in the central nervous system of the rat In *International symposium on amphetamines and related compounds* Ed E. Costa & S Garattini Raven Press New York, 1970, 301-316

Hanson, L. C F Evidence that the central action of (+)- amphetamine is mediated via catecholamines *Psycho-pharmacologia (Berl)* 1967, 10 289-297

Menon M K & P C Dandiya Mechanism of the protective effect of reserpine on aggregated mice treated with (+)-amphetamine *J Pharm Pharmacol* 1967, 19 596–602

Mirkin B L. Developmental pharmacology *Ann Rev Pharmacol* 1970, 10 255-272

Randrup A & J Munkvad Role of catecholamines in the amphetamine excitatory response *Nature (Lond)* 1966, 211, 540

Weissman, A, B K Koe & S Tenen Antiamphetamine effects following inhibition of tyrosine hydroxylase *J Pharmacol exp Therap* 1966, 151, 339–352.

Acta pharmacol et toxicol 1973, 32, 129–138

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On the Elimination of 5-Hydroxyindoleacetic Acid and Homovanillic Acid from Cerebrospinal Fluid

By

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Abstract The effect of carinamide, probenecid and zoxazolamine was studied in the brain and cerebrospinal fluid of the dog rabbit and rat There were con siderable differences between these species with regard to the effectiveness of the active out transport mechanism for acid monoamine metabolites

Key words Cerebrospinal fluid – 5 hydroxyindoleacetic acid – homovanillic acid – animals

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There are three ways for the elimination of acid monoamine metabolites from the cerebrospinal fluid (CSF), viz. bulk flow, diffusion and active transport Very little is still known about the relative importance of these processes (ASHCROFT *et al* 1968, MOIR *et al* 1970) Bulk flow being a passive process, whereas the metabolites pass out together with CSF, provided the molecules are not too large, is not influenced by the physical properties of the compounds transported Diffusion from the CSF takes part in the whole ventricular system but mainly in the subarachnoid space (DAVSON 1967) The first step might be the diffusion into the brain parenchyma and the second one, from the brain parenchyma into the blood It is also likely that diffusion takes place directly from the CSF to blood vessels in the subarachnoid space Of importance for the diffusion in addition to the requirement of a suitable concentration gradient is the lipid solubility of the substance The more lipid soluble a compound, the easier is the diffusion For active transport, the concentration gradient between the two mediums is not rate limiting since most of these transports seems to be carrier mediated Thus it is possible for two substances using the same transport mechanism to compete with each other The purpose of this investigation was to clarify the relative importance of the different ways of elimination for 5 hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) from the central nervous system (CNS) and the CSF in various animal species

There is an important difference in the physical properties of the two compounds, 5-HIAA and HVA The 5-HIAA molecule is less lipid soluble than the HVA This appears e g in the methods of determination While HVA can be extracted from a water phase adjusted to about pH < 1 into a rather non polar organic solvent, e g heptane, 5 HIAA has to be extracted from a water phase, which has to be both acid and saturated with sodium chloride Both 5-HIAA and HVA cross the blood brain and blood CSF barriers poorly, but whereas 5-HIAA does not cross these barriers in doses of 4–5 mg per kg intravenously, this occurs with HVA In order to investigate the possible differences in the pattern of elimination of the two acids, we have used probenecid, carinamide and in some experiments zoxazolamine, which is not an acid but known to be a good blocker of the reabsorption of acids in the distal tubules of the kidney (BURNS *et al* 1958)

Material and Methods

All the experiments including sampling of the CSF were performed under general anaesthesia with pentobarbital (mebumalum NFN) The animals were allowed to breathe spontaneously In the experiments where monoamines and their acid meta bolites in the brain were determined after no treatment or at various intervals after the administration of drugs the rabbits were killed by air embolism and the rats by decapitation The experimental models regarding drugs doses and methods for sampling of CSF and investigations on the brain were different in different species. They will therefore be described separately

Dogs In one series of experiments carinamide (*p*-(benzylsulphonamido)-benzoic acid) in physiological saline was injected intravenously at the beginning of the experiment (100 mg/kg) In another series of experiments carinamide was injected three times intra cisternally at the beginning of the experiment at 60 and 120 min (1 mg dissolved in 2 ml of physiological saline each time) The CSF was collected by percutaneous cisternal puncture and 2 ml were sampled each time The time intervals for sampling are scheduled in fig 2 and 3

In another series of experiments dogs were injected intravenously with probenecid 50–200 mg/kg The CSF was sampled both by ventricular and cisternal puncture The time is scheduled in fig 4 The brains were removed at different intervals after the injection of the drugs In some experiments the brains were hemisected sagittally and in one of the halves the caudate nucleus and brain stem were investigated for 5 HIAA and HVA (table 1) The other half of the brain was used for determinations of the total concentrations of 5 HIAA HVA 5 hydroxytryptamine (5 HT) noradrenaline (NA) and dopamine (DA) (table 1)

Rabbits 1) Carinamide was injected intravenously in doses ranging 25–100 mg/kg body weight CSF was sampled and the brain taken for analyses at two or four hours after the intravenous injection of the drug (table 2)

2) Probenecid was injected intravenously 200 mg/kg The CSF was sampled by cisternal puncture two or four hours after administration of the drug (table 2)

Rats 1) Carinamide was injected intraperitoneally 200 mg/kg and the animals were killed four hours after the injection The brains were removed and the whole brain was used for analyses (table 3)

Table 1

The effect of probenecid intracisternally and intravenously, on HVA, DA, 5 HIAA, 5 HT and NA in caudate nucleus, brain stem and whole brain of the dog

Probenecid given intracisternally 2 mg × 3 Brain analysis 5–7 hrs after the original injection Probenecid given intravenously 50–100 mg per kg Brain analysis 4–7 hrs after the drug Values are expressed in ng per gram tissue ± S E M (n) No significant differences.

	HVA	DA	5 HIAA	5 HT	NA
Controls					
Caudate nucleus	15915 ± 906 (9)	n. d	468 ± 29 (4)	n d	n d
Brain stem	329 ± 72 (4)	n d	1169 ± 120 (11)	n d	n d
Whole brain	991 ± 11 (3)	201 ± 4 (3)	296 ± 27 (3)	189 ± 12 (3)	154 ± 10 (3)
After probenecid i c					
Caudate nucleus	20738 ± 1454 (3)	n d	n d	n d	n d
Brain stem	n d	n d	1149 ± 257 (3)	n. d	n d
Whole brain	892 ± 56 (3)	224 ± 20 (3)	327 ± 71 (3)	209 ± 30 (3)	198 ± 23 (3)
After probenecid i v					
Caudate nucleus	16424 ± 1369 (9)	n d	386 ± 72 (5)	n d	n d.
Brain stem	346 ± 27 (5)	n d	994 ± 88 (9)	n. d	n d
hole brain	1025 ± 87 (6)	219 ± 37 (4)	360 ± 45 (6)	220 ± 25 (4)	204 ± 43 (4)

2) Zoxazolamine, 20–100 mg/kg dissolved in oil of arachic was given orally and the animals were killed four hours after administration of the drug. The brains were removed and the whole brain was used for analyses (table 3).

3) Probenecid was injected intraperitoneally, 200 mg/kg, and the cisternal puncture was done two hours later, after which the brain was removed for analyses. CSF-samples were pooled (15–25 animals) (table 3).

HVA was measured in the CSF and brain tissue according to KORF *et al.* (1971) and to ANDÉN *et al.* (1963). In the CSF, 5-HIAA was determined according to SHARMAN (1960) and in brain tissue according to JOHNSSON & LEVANDER (1970). 5-HT in brain was determined according to ANDÉN & MAGNUSSON (1967). DA according to CARLSSON & LINDQVIST (1962) and NA according to BERTLER *et al.* (1958). Carinamide and probenecid were measured in the plasma and CSF according to modifications of the methods described for carinamide by BRODIE *et al.* (1947) and for probenecid by TILLSON *et al.* (1954).

Result

Dogs. Two and a half hours after the intravenous injection of carinamide there was still no detectable concentration of the drug in the CSF. At the same time, however, there was a slow but significant increase in HVA and 5-HIAA (fig. 2). The level of carinamide in the plasma declined rapidly and the half life was calculated to be 90 min. (fig. 1). After the intracisternal injection, on the contrary, the values of the monoamine acids in the cisterna increased faster and higher and to equal levels. The elimination of carinamide from the CSF was rapid (fig. 3). In brain tissue there was no significant increase in the monoamines or their corresponding acid metabolites either in the whole brain, or in the brain stem and caudate nucleus. The brain stem was defined according to ANDERSSON & ROOS (1968).

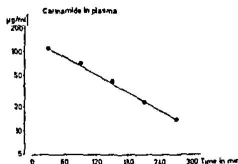


Fig. 1. Concentration of carinamide in dog plasma after 100 mg per kg intravenously. Values are µg per ml.

Probenecid given intravenously 50–200 mg per kg caused a parallel increase in 5 HIAA and HVA in the ventricle (fig 4) These experiments also showed that the maximum concentration of probenecid was obtained in the lateral ventricle (fig 4)

Rabbits The intravenous injection of carinamide caused a significant increase in the concentration of 5-HIAA in CSF with its maximum after 2 hours (table 2) There was no change in the concentration of HVA in the CSF Analyses of the brain stem for 5-HIAA and caudate nucleus for HVA showed no change either at 2 or 4 hours after the injection (table 2)

There was a significant increase in both acids in the CSF 2 hours after 200 mg per kg probenecid (table 2) In whole brain HVA was increased significantly after 2 hours, but 5-HIAA did not increase until 4 hours after the drug This increase in 5-HIAA remained for 8 hours after the injection (table 2)

Rats Carinamide intraperitoneally into the rat had no effect in whole brain

Zoxazolamine given orally in doses exceeding 20 mg per kg produced a significant increase of 5 HIAA in brain tissue but no change in the level of HVA (table 3)

After probenecid the CSF values of 5-HIAA increased about three times There was no measureable concentration of HVA in the CSF in untreated animals and the eventual rise after the administration of the drug was not significant (table 3) However, the values of both metabolites increased significantly in brain tissue (table 3)

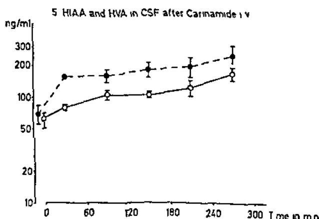


Fig 2 Intravenous injection of carinamide 100 mg per kg to the dog at time zero 5 HIAA open circles and HVA closed circles in cisternal CSF are in ng per ml ± S D (2) The carinamide induced increase in the acids is still demonstrable 300 min after the injection the values do not reach the base line

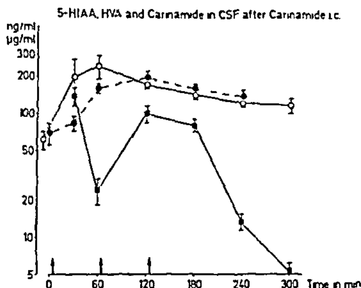


Fig 3 Intracisternal injection to the dog of carinamide 1 mg at arrows 5 HIAA open circles and HVA closed circles are in ng per ml ± S E M (4) Carinamide squares, are in ng per ml ± S E M

Table 2

The effect of carinamide and probenecid on HVA and 5 HIAA in CSF and brain of the rabbit

Carinamide 25–100 mg per kg intravenously to mongrel rabbits Value in CSF are ng per ml ± S E M (n) Values in brain are 5 HIAA in brain stem HVA in caudate nucleus – ng per g tissue ± S E M (n)

Probenecid 200 mg per kg intravenously to white rabbits Values in CSF are ng per ml ± S E M (n) Values in brain are from whole brain for both 5-HIAA and HVA ng per g tissue ± S E M (n) Significant differences from controls are marked with asterisks.

	CSF		Brain stem	
	HVA	5 HIAA	HVA	5 HIAA
Controls	37 ± 8 (3)	122 ± 3 (3)*	3612 ± 351 (8)	842 ± 69 (8)
Carinamide				
2 hours	47 ± 4 (2)	241 ± 5 (4)	3953 ± 360 (7)	779 ± 82 (7)
4 hours	40 ± 5 (2)	157 ± 10 (5)*	3636 ± 541 (6)	940 ± 97 (5)
Controls	42 ± 1 (3)	203 ± 17 (8)	415 ± 49 (4)	473 ± 6 (4)
Probenecid				
2 hours	400 ± 85 (3)*	717 ± 65 (11)*	705 ± 44 (4)*	491 ± 36 (4)
4 hours	n d	541 ± 35 (7)*	862 ± 130 (4)*	621 ± 59 (4)*
8 hours	352 (1)	419 ± 109 (3)	597 ± 71 (4)	617 ± 41 (4)*
16 hours	100 ± 37 (2)	124 ± 12 (3)	339 ± 5 (2)	530 ± 92 (2)

Discussion

Carinamide The poor effect of the intravenous treatment with carinamide in the dog is apparently due to the slow increase of the drug in the CSF This may probably be due to the rapid elimination of carinamide from both the plasma and CSF, where the half life is 90 min and less than 30 min, respectively On the contrary, when the level of carinamide in the CSF is held on a sufficient level by repeated intracisternal injections, the HVA and 5-HIAA continue to increase simultaneously, attaining about the same level The importance of a sufficient concentration of the blocking drug in the CSF is thus apparent c f probenecid

In the rabbit there is only an increase of 5-HIAA in the CSF but not of HVA The dog brain was not investigated after carinamide, but both in rabbits and rats there was no change in the acids

It is therefore obvious that the only effect of carinamide is on the transport from the CSF to the blood

Probenecid After probenecid intravenously to the dog both acids increase in parallel in the cisterna and ventricle Probenecid itself shows a rapid increase in both compartments, but the concentration is higher in

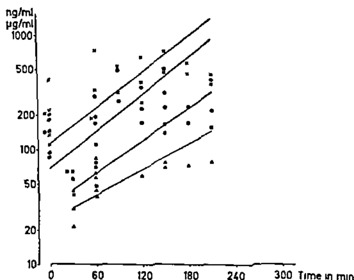


Fig 4 Probenecid intravenously to dog 50–200 mg per kg The regression lines counted from top are as follows HVA in ventricular CSF (crosses) r = 0 614 5 HIAA in ventricular CSF (dots) r = 0.566 Probenecid in ventricular CSF (squares) r = 0 831 Probenecid in cisternal CSF (triangles) r = 0 698 The values of HVA and 5 HIAA are in ng per ml and the values of probenecid in µg per ml

Table 3

The effect of carinamide, probenecid and zoxazolamine on 5 HIAA and HVA in whole brain and CSF of the rat

Values of 5 HIAA and HVA in CSF ng per ml ± S E M (n) and whole brain tissue ng per g ± S E M (n) The drugs were given carinamide intraperitoneally 200 mg per kg to black hooded rats probenecid intraperitoneally 200 mg per kg to white Wistar rats zoxazolamine orally 20–40 and 60–100 mg per kg to white Wistar rats Significant differences from controls are marked with asterisks

	Whole brain		CSF	
	5 HIAA	HVA	5 HIAA	HVA
Controls	513 ± 15 (6)	32 ± 2 (6)	n d	n. d
Carinamide 4 hours	493 ± 11 (4)	26 ± 1 (6)	n d	n. d.
Controls	379 ± 2 (2)	45 ± 2 (8)	271 ± 65 (2)	0 (2)
Probenecid 2 hours	792 ± 28 (6)*	134 ± 7 (9)*	724 ± 90 (2)*	35 ± 35 (2)
Controls	221 ± 3 (8)	33 ± 1 (6)	n d	n. d
Zoxazolamine 4 hours				
20–40 mg	354 ± 27 (9)*	n d	n d.	n. d
60–100 mg	358 ± 26 (13)*	37 ± 3 (5)	n d	n d

the lateral ventricle, which implies that probenecid probably enters the CSF via the chorioid plexus

In the rabbit there is an increase of both acids in the CSF but the percentual increase of HVA is much higher than that of 5-HIAA in spite of the HVA level in rabbit CSF being very low This will be discussed later In the rat there is only an increase in the CSF-5-HIAA HVA does not reach measurable values in the CSF of this animal

In the dog probenecid has no detectable effect on the content of amines and acid metabolites in the brain In the rabbit on the contrary there is an increase in the metabolites In whole brain of the rabbit the levels of the metabolites are nearly equal, but the increase after probenecid is more marked for HVA The most plausible explanation for this, is a higher rate of turnover for HVA As a consequence of this there is an increased inflow of HVA in the CSF The same pattern is seen in the rat brain, where the percentual increase of HVA is still more pronounced In contrast to the dog and the rabbit it seems as if the rat does not use CSF at all for the transport

of HVA Of course, the possibility of an extremely good capacity for the elimination of HVA cannot be excluded

Zoxazolamine, finally, which in contrast to the other two compounds is a weak base, increases the level of 5-HIAA in the brain tissue of the rat but not of HVA This might support earlier assumptions that 5-HIAA and HVA at least partly have different pathways of elimination

Compared to probenecid carinamide is a less effective blocker of the active transport from the CSF It still has any effect on 5-HIAA transport in the rabbit CSF and a very small effect on both acids in the dog This supports the hypothesis that 5-HIAA is more dependent on the active transport mechanisms

In whole brain of the rabbit there is an equal amount of 5-HIAA and HVA, but in the dog the total concentration of HVA in whole brain is four times higher than that of 5-HIAA Thus, in all evaluations of the data on the effects of different blockers on the active transport mechanism for weak acids, e g 5 HIAA and HVA from the CSF or CNS to the blood, one has to consider the animal species investigated

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REFERENCES

Anden N E, B E Roos & B Werdinius The occurrence of homovanillic acid in brain and cerebrospinal fluid and its determination by a fluorimetric method *Life Sci* 1963, 2 448–458

Anden N E & T Magnusson An improved method for the fluorimetric determination of 5 hydroxytryptamine in tissues *Acta physiol scand* 1967 69 87–94

Andersson H & B E Roos 5 Hydroxyindoleacetic acid in ventricular cerebrospinal fluid and brain of normal and hydrocephalic dogs after administration of 5 hydroxy tryptophan *Acta pharmacol et toxicol* 1968, 26, 531–538

Ashcroft G W R C Dow & A T B Moir The active transport of 5 hydroxyindole 3 ylacetic acid and 3 methoxy-4-hydroxyphenylacetic acid from a recirculatory per fusion system of the cerebral ventricles of the unanaesthetized dog *J Physiol* 1968 199, 397–425

Bertler A, A Carlsson & E. Rosengren A method for the fluorimetric determination of adrenaline and noradrenaline in tissues *Acta physiol scand* 1958 44 273–292

Brodie, B B, B Levy & E Bernstein The estimation of 4-carboxyphenylmethane sulfonanilide (caronamide) in biological fluids *J Pharmacol exp Therap* 1947, 91, 246–249

Burns J J, T F Yu, L. Berger & A B Gutman Zoxazolamine physiological disposition uricosuric properties *Amer J Med* 1958, 25, 401–408

Carlsson, A. & M Lindqvist *In vivo* decarboxylation of α methyl DOPA and α methyl metatyrosine *Acta physiol scand* 1962, 54 87–94

Davson H *Physiology of the cerebrospinal fluid* 1st ed J & A. Churchill, London 1967, pp 165–183

Jonsson, J & T Levander A method for the simultaneous determination of 5 hydroxy 3 indoleamine (5 HIAA) and 5 hydroxytryptamine (5 HT) in brain tissue and cerebrospinal fluid *Acta physiol scand* 1970, 78 43–51

Korf, J, B-E Roos & B Werdinius Fluorimetric determination of homovanillic acid in tissues, using anion exchange separation and mixed solvent elution *Acta Chem Scand* 1971, 25, 333–335

Moir, A T B, G W Ashcroft T B B Crawford, D Eccleston & H C. Guldberg Cerebral metabolites in cerebrospinal fluid as a biochemical approach to the brain *Brain* 1970, 93, 357–368

Sharman, D F *The significance of pharmacologically active amines in animal tissues and body fluids* Thesis Edinburgh, 1960

Tillson, E K, G S Schuchardt, J K. Fishman & K. H Beyer The determination of probenecid (benemid) in body fluids *J Pharmacol exp Therap* 1954, 111, 385–394

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5-Hydroxyindole Acetic Acid and Homovanillic Acid in Cerebrospinal Fluid after Intrathecal and Intravenous Administration of Probenecid to Normal and Hydrocephalic Dogs

By

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(Received June 23, 1972, Accepted July 20, 1972)

Abstract This study has shown that intrathecal instillation of probenecid gives a lower elevation of the acid metabolites than intravenous administration of the drug, providing the concentration of the drug in the cerebrospinal fluid is the same Furthermore, we have reason to believe that it is possible to block almost completely the active out transport of 5 hydroxyindole acetic acid from the cerebrospinal fluid by high doses of probenecid given intravenously or intrathecally This does not seem to be the case with homovanillic acid

Key words 5 Hydroxyindole acetic acid – homovanillic acid – cerebrospinal fluid – probenecid – hydrocephalic dogs

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Probenecid blocks the transfer of 5-hydroxyindole acetic acid (5-HIAA) from brain to blood in the rat (NEFF *et al* 1964 & 1967), and also the transfer of homovanillic acid (HVA) (WERDINIUS 1966) Probenecid blocks the egress of these acid monoamine metabolites from the cerebrospinal fluid (CSF) to the blood (GULDBERG *et al* 1966)

Some organic acids when incorporated in a ventriculocisternal perfusion system are transferred from the CSF by means of an active transport mechanism similar to the secretion in the proximal tubules of the kidney (PAPPENHEIMER *et al* 1961, DAVSON *et al* 1962, POLLAY & DAVSON 1963) The specific out transport mechanism is believed to be localized in the region of the fourth ventricle (PAPPENHEIMER *et al* 1961)

The intravenous infusion of probenecid retards the elimination of intraventricularly injected 5-HIAA from the CSF in normal dogs, but has no effect on the elimination of 5-HIAA in dogs, when hydrocephalus has been induced with intracisternally injected kaolin (ANDERSSON & ROOS 1968) The probenecid sensitive transport mechanism does not seem to work in the hydrocephalic dog perhaps as a consequence of the elevated intracranial

pressure There may be a parallelism with the tubular function in the kidney, which is disturbed in hydronephrosis

It has generally been assumed that probenecid should be given orally or intravenously in order to produce the desired blood concentration It is possible that probenecid acts as a competitive inhibitor of the mechanism for eliminating similar organic acids

The main purpose of this study was to find out, whether probenecid administered intrathecally has the same effect on the acidic metabolite levels as intravenously injected probenecid Further it was important to know, whether it is possible to block completely the active out transport mechanism with probenecid

Material and Methods

Mongrel dogs weighing 5–15 kg under pentobarbital (mebumalum NFN) anaesthesia with spontaneous breathing were used in these studies In normal animals CSF was collected by percutaneous cisternal puncture and in hydrocephalic dogs by ventricular puncture after a small craniotomy Hydrocephalus was induced by intracisternal injection of 2 ml of a 40 % kaolin suspension 2 3 weeks before the experiments Two ml of CSF was withdrawn on every occasion for the estimation of probenecid 5 HIAA and HVA Probenecid was injected intravenously at the beginning of the experiment after 120 and 240 min (100 mg/kg each time) In another series of experiments probenecid was injected intrathecally (1 mg and 4 mg dissolved in 2 ml of physiological saline each time) at the beginning of the experiment after 60 and 120 min In the experiments where probenecid was injected intravenously as well as intrathecally this was done after withdrawal of 2 ml of CSF The elimination rate of probenecid from cisternal CSF was *calculated by observations for four hours of the decline of the probenecid* values after a single intracisternal injection of 1 mg of the drug

HVA was measured in CSF (KORF *et al* 1971) 5 HIAA was measured in CSF (SHERMAN 1960) Probenecid both in the plasma and CSF was measured by a modified spectrophotometric method based on TILLSON *et al* (1954) and DAYTON *et al* (1963)

Results

In normal dogs there was a three fold increase in the cisternal values of HVA and 5 HIAA one hour after the administration of 1 mg probenecid intrathecally (fig 1a) One hour after the administration of 4 mg the HVA *value had increased three-fold (fig 1b) Repeated intrathecal* instillation of probenecid caused a further increase of the HVA and 5 HIAA levels After instillation of probenecid into the ventricle of a hydrocephalic dog there was no increase in the ventricular values of 5 HIAA and HVA Further sampling of CSF showed a gradual decrease of the values (fig 2) When probenecid was injected intravenously the acid monoamine metabolites in the cisternal

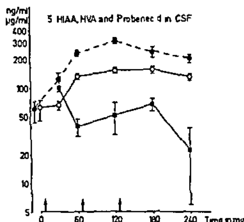


Fig 1a 5 HIAA (ng/ml ± S E M , open circles) and HVA (ng/ml ± S E M , closed circles) after intrathecal administration of 1 mg probenecid (at arrows to normal dogs (n=6) Semi logarithmic diagram Probenecid in µg/ml (closed squares)

CSF increased continuously. There was also a parallelism between the increase of these acids and the increase in the concentration of probenecid in the CSF (fig 3) However, the animals died when the plasma level of probenecid exceeded 450 µg/ml

The positive correlation between the levels of probenecid in plasma and CSF after the intravenous injection is shown in fig 4 The half life of probenecid in cisternal CSF after intrathecal administration was calculated to be 35 min

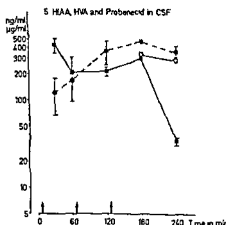


Fig 1b 5 HIAA (ng/ml ± S D , open circles) and HVA (ng/ml ± S D closed circles) after intrathecal administration of 4 mg probenecid (at arrows) to normal dogs (n=2) Semi logarithmic diagram. Probenecid in µg/ml (closed squares)

per ml in contrast to the value in the normal dog after probenecid, which is about 700 ng per ml These findings suggest that the elimination is completely blocked for 5-HIAA but not for HVA after intravenously tolerable doses of probenecid The drug is very toxic in doses exceeding 300 mg/kg intravenously

When increasing the CSF probenecid by the intrathecal administration of the drug, the increase in the concentration of probenecid in the CSF shows a continuous rise in both 5-HIAA and HVA, but the values reach different levels Normally the values of 5-HIAA and HVA in cisternal CSF are of the same magnitude After intrathecal as well as intravenous injection of probenecid the HVA value in cisternal CSF increases twice as much as the value of 5-HIAA (fig 1 and 3) In hydrocephalus the same difference is encountered in the ventricular CSF without probenecid (fig. 2) This might at least partly depend on the more rapid turnover rate for DA than for 5 HT in nervous tissue The concentration of HVA in caudate nucleus is about 15000 ng per g brain tissue and in ventricular CSF about 200 ng per ml Furthermore when the active transport mechanism is totally blocked and CSF-HVA is increased to about 700 ng per ml (fig 3), there is still a steep concentration gradient between brain tissue and CSF allowing an undisturbed diffusion of HVA from the striatal structures into the CSF On the contrary, when 5-HIAA in CSF after probenecid or in hydrocephalic animals reaches a value of 400 to 500 ng per ml, the difference in concentration between CSF and brain is very small In the parts of brain immediately adjacent to the ventricular system values of 5-HIAA do not exceed 1000 ng per g tissue. Our present data are not sufficient to decide whether this is the main explanation or whether the described differences between 5 HIAA and HVA in the utilization of diffusion and active transport are of almost the same importance Investigations now in progress will probably show the quantita 'e changes in nervous tissue after probenecid

DAYTON *et al* (1963) gave probenecid 40 mg per kg intravenously to dogs d obtained plasma concentrations of 139–194 μg per ml The half life was 6 hours This corresponds to the data of TILLSON *et al* (1954) We found at after the intravenous injection there was a positive correlation between the concentrations of probenecid in the plasma and CSF (fig 4) This was, however, not directly proportional in a way associated with a direct diffusion of free probenecid from the plasma to CSF Probenecid probably passes into CSF via the choroid plexuses but it may also gain access to CSF by diffusion from brain parenchyma When we investigated the half life of probenecid in CSF, it was found to be extremely short, i e about half an hour The rapid elimination of probenecid from CSF might at least partly also be responsible for the slow accumulation of the drug in the CSF after repeated intravenous injections

We were trying to obtain a concentration of probenecid in CSF sufficient for a complete block of the active transport of 5-HIAA and HVA In order to reach the optimal level we gave repeated doses of probenecid intravenously with 2 hours' interval, which resulted in a plasma concentration of about 350 μg per ml and a concentration in CSF around 50 μg per ml If the plasma concentration exceeded 450 μg per ml, toxic symptoms occurred and the dogs died We also gave the drug intrathecally in a dose of 1 mg × 3 producing about the same concentration in CSF as obtained by intravenous administration

There was, however, a definite difference between the results obtained by intrathecal and intravenous administration of probenecid, respectively Even if the levels of probenecid in CSF were about the same in the two experimental conditions, the levels of HVA and 5-HIAA after intrathecal administration were only half the levels obtained by the intravenous route (fig 1 and 3) This might be due to a different magnitude of the block caused by the different ways of administration Probenecid given intravenously to rabbits causes a block of the actice transport both from the brain tissue and CSF to blood resulting in an increased concentration of the acid monoamine metabolites in brain leading to enhanced levels of the acids in CSF (ANDERSSON & ROOS 1972) Probenecid intrathecally probably blocks only the active transport from CSF to blood. There has been continuous discussion around the out-transport of acids from the brain to blood versus brain to CSF It is not known whether the quotient between these two ways is constant or changes according to treatment with various drugs The relative importance of these two ways can of course be different in different species Probenecid given intravenously blocks both mechanisms Probenecid given intrathecally, however, only impairs the egress from CSF to blood This provides a method for studying these conditions

It is not possible to increase the CSF level of probenecid to more than 50 μg per ml by the intravenous administration of the drug After intrathecal instillation of 4 mg × 3, however, a level of 400 μg per ml was reached In spite of this very high concentrations the values of 5-HIAA and HVA did not reach a level higher than that observed after intravenous administration The shapes of the two curves for 5 HIAA, HVA and probenecid after 1 mg × 3 and 4 mg × 3 were similar

When the value for 5 HIAA was close to that seen in hydrocephalus, the HVA level did not increase to more than a third of the hydrocephalus level

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REFERENCES

Andersson, H & B E Roos The effect of probenecid on the elimination from CSF of intraventricularly injected 5 hydroxyindoleacetic acid in normal and hydrocephalic dogs *J Pharm Pharmacol* 1968, 20, 879–887

Andersson, H & B E Roos 5 Hydroxyindoleacetic acid and homovanillic acid in cerebrospinal fluid and brain of different rabbit breeds after treatment with probenecid. *J Pharm Pharmacol* 1972, 24, 165–166

Davson, H *Physiology of the cerebrospinal fluid* 1st ed, J & A Churchill, London 1967, p 165–183

Davson, H, C. R. Kleeman & E Levin Quantitative studies of the passage of different substances out of the cerebrospinal fluid *J Physiol* 1962, 161, 126–142.

Dayton P G., T F Yu, W Chen, L. Berger, L. A West & A B Gutman The physiological disposition of probenecid, including renal clearance, in man, studied by an improved method for its estimation in biological material *J Pharmacol exp Therap* 1963, 140 278–286

Guldberg H C, G W Ashcroft & T B B Crawford Concentrations of 5 hydroxyindoleacetic acid and homovanillic acid in the cerebrospinal fluid of the dog before and during treatment with probenecid *Life Sci* 1966 5, 1571–1575

Korf J B E Roos & B Werdinius Fluorometric determination of homovanillic acid in tissues using anion exchange separation and mixed solvent elution *Acta Chem Scand* 1971, 25 333–335

Neff N H, T N Tozer & B B Brodie A specialized transport system to transfer 5 HIAA directly from brain to blood *Pharmacologist* 1964 6 162

Neff, N H T N Tozer & B B Brodie Application of steady state kinetics to studies of the transfer of 5 hydroxy indoleacetic acid from brain to plasma *J Pharmacol exp Therap* 1967, 158 214–218

Pappenheimer J R S R Heisey & E F Jordan Active transport of diodrast and phenolsulfonphtalein from cerebrospinal fluid to blood *Amer J Physiol* 1961, 200 1–10

Pollay, M & H Davson The passage of certain substances out of the cerebrospinal fluid *Brain* 1963, 86 137 150

Sharman D F *The significance of pharmacologically active amines in animal tissues and body fluids* Thesis Edinburgh, 1960

Tagliamonte, A, P Tagliamonte, R Gessa, M Duce, C Maffei & G L. Gessa Increase of brain tryptophan by probenecid *Riv Farmacol Ter* 1971, II 207–213

Tillson, E K G S Schuchardt, J K. Fishman & K H Beyer The determination of probenecid (Benemid) in body fluids *J Pharmacol exp Therap* 1954, **111**, 385–394
Werdinius, B Effect of probenecid on the level of homovanillic acid in the corpus striatum *J Pharm Pharmacol* 1966, **18**, 546–547

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The Effects of Promazine and Thioridazine on the Response to Noradrenaline in Isolated Rat Atria

By

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(Received June 26, 1972, Accepted August 17, 1972)

Abstract The effects of promazine and thioridazine on contractile force and rate, and on the response to noradrenaline (NA) have been studied in isolated spontaneously beating and electrically stimulated rat atria. Promazine 5×10^{-6} M increased the contractile force, while promazine 2.5×10^{-5} M reduced it Correspondingly 2.5×10^{-6} M promazine increased the rate of contraction, while 10^{-5} M reduced it. Only depressive effects were obtained with thioridazine 2.5×10^{-6} M – 2.5×10^{-5} M, and these were less marked than those produced by promazine when the highest concentrations were compared Both drugs enhanced the effect of low concentrations (5×10^{-7} M) of NA on the contractile force and reduced the effects of higher concentrations (The effects of promazine were statistically significant ($P < 0.05$), but not those of thioridazine) Promazine had the same effect on the alterations in rate produced by NA. Thioridazine on the other hand, while reducing the response to the highest concentrations of NA had no enhancing effect on the increase in rate produced by lower concentrations It is suggested that the enhancing effect of the phenothiazines is due to a reduced uptake of NA in sympathetic nerve endings while the cardio-depressive effects are due to the membrane stabilizing action on the myocardium

Key words Phenothiazines – noradrenaline – isolated rat atria.

The effects of promazine and thioridazine on isolated rat atria have been extensively studied in this laboratory (LANDMARK 1971a, 1971b, 1972a & 1972b, LANDMARK *et al* 1972) These studies show that the two phenothiazines depress atrial activity by a direct action on the myocardium. The studies, were carried out *in vitro*, however, and it is uncertain to what extent the same effects could be obtained *in vivo* In the living animal the effect of a drug depends not only on its direct effect on the organ studied, but also on its effect on the regulating influences which normally control the function of that organ In order to obtain more information about how promazine and thioridazine affect the nervous control of cardiac activity, we have previously

(HAFFNER & LANDMARK 1972) studied the interaction between acetylcholine and the two phenothiazines, in an attempt to determine their influence on the cholinergic innervation The present study which concerns the effects of promazine and thioridazine on the response to noradrenaline (NA) in isolated rat atria, was undertaken in order to determine how the two phenothiazines influence the adrenergic control of the heart

Material and Methods

Female albino Wistar rats weighing approximately 200 g were anaesthetized with ether, the hearts were excised and the atria removed by dissection The experiments with spontaneously beating atria were performed with "double atria , i e left and right atria dissected out "en block", while the experiments with electrically stimulated preparations were performed with left atria The preparations were tied to a Perspex holder, and mounted in an organ bath containing 30 ml modified Ringer solution kept at either 32° or 38° The Ringer solution had the following composition (ions in meq /l) NA^{+} 143 4 K^{+} 5 3 CA^{++} 5 1 Mg^{++} 2 3 Cl 126 4 H_2PO_4 2 4, HCO_3 25 SO_4^{--} 2 3 The solution also contained glucose 1 8 mg/ml It was bubbled with 95 % O_2 and 5 % CO_2 which gave a pH of 7 4 A preload of 400 mg was put on the preparations and the contractile force was recorded isometrically with a Grass force displacement transducer (FT03C) connected to a Grass polygraph (Model 7WC12PA) The single, left atria were stimulated electrically through platinum electrodes at a frequency of 180 per min by square wave pulses of 0.5 msec duration, delivered by a Biotronic laboratory stimulator The voltage used was 3 times the threshold as it was found that otherwise some preparations failed to follow the stimulation after treatment with promazine and thioridazine The threshold varied from 0 6–3 0 V, measured on a Tektronix dualbeam oscilloscope type 502A

After an equilibration period of 30 min the contractile force and rate were determined (=100 %) and 15–150 µl solution of phenothiazine or 50 µl deionized water (controls) was added to the bath. The phenothiazines (promazine and thioridazine) were added in amounts which gave final bath concentrations of 2 5×10^{-6} 5×10^{-6} 10^{-5}

without washing between successive additions The maximal effect of each concentration of NA was determined before the next concentration was added the time interval between additions varying from 1¼ to 1¾ min The cumulative dose response curve was used in the present investigation because preliminary studies showed that it was impossible to wash out the effect of NA completely It was therefore not feasible to carry out two successive dose response curves on each preparation, as done in the previous investigation with ACh

All results are expressed as per cent of the values obtained at the end of the equilibration period (100 %) The Wilcoxon two-sample test was used to test the statistical significance of the difference in response in the control group and the test group (The test is based on ranking with numbers from 1 upwards of all the results in the control group (s) and the test group (t) according to magnitude of response)

The *drugs used* were promazine HCl (Norfarma) thioridazine HCl (Sandoz) and

noradrenaline bitartrate The solutions of NA were prepared from a frozen stock solution while fresh solutions of the phenothiazines were prepared each day

Results

Contractile force

Noradrenaline (NA) increased the contractile force in the electrically stimulated atria, and a dose response curve could be worked out for this effect (fig 1)

Promazine 5×10^{-6} *M* caused a slight, but statistically significant (s=t=6, P < 0 05) increase in initial contractile force as compared with the controls (fig 1) A corresponding increase was found in the responses to 2×10^{-8}, 10^{-7} and 5×10^{-7} M NA The difference in contractile force from the control group is statistically significant for 2×10^{-8} M NA (s=t=6, P < 0 01), but *not for the other concentrations of NA*

Promazine 10^{-5} *M* had very little direct effect on contractile force, but reduced the responce to NA The reduction was most marked at the highest concentrations of NA, and as seen in fig. 1, the dose response curve to NA *slopes downward from* 5×10^{-7} *to* 10^{-5} *M in the presence of* 10^{-5} *M promazine* The reduction in the response to NA is statistically significant only for NA 10^{-5} M (s=t=6, P < 0 05)

Promazine 2.5×10^{-5} *M* caused a great reduction in initial contractile force

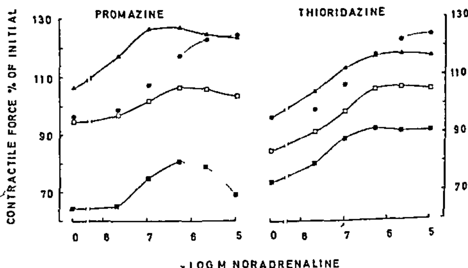


Fig 1 Log dose response curves for the effect of NA on contractile force in isolated electrically stimulated rat atria treated with promazine or thioridazine ● control ▲— 5×10^{-6} M □— 10^{-5} M ■— 2.5×10^{-5} M, (Dotted line two atria which failed to follow stimulation are not included) Each curve represents the mean of 6 experiments For statistical evaluation see text

(mean reduction=35 8 %, s=t=6, $P<0\ 002$), and the contractile force after addition of NA was correspondingly reduced ($P<0\ 002$ for all points on the dose-response curve) Two out of 6 preparations developed arrhythmia when exposed to 2×10^{-6} M and 10^{-5} M NA after treatment with $2\ 5\times10^{-5}$ M promazine

It should be noted that promazine had two apparent effects on the dose-response curve for NA in the electrically stimulated atria Promazine 10^{-5} M, which had no direct effect on the contractile force, produced a slight flattening of the dose response curve, the other two concentrations altered the curve in roughly the same way, but also shifted it parallelly upwards (5×10^{-6} M) or downwards ($2\ 5\times10^{-5}$ M)

Thioridazine 5×10^{-6} *M* had no apparent effect on the contractile force but enhanced the effect of 2×10^{-8} and 10^{-7} M NA slightly, and caused a slight reduction in the effect of 2×10^{-6} and 10^{-5} M NA (fig 1) These alterations in the dose response curve were too small to be statistically significant (s=t=6, $P>0\ 1$), but it should be noted that they are of the same character as those produced by promazine

Thioridazine 10^{-5} *and* $2\ 5\times10^{-5}$ *M* altered the dose response curve in the same way, and as these concentrations reduced initial contractile force markedly the curve was shifted parallelly downwards (see fig 1) The mean reduction in the initial contractile force produced by thioridazine 10^{-5}M was 9 1 %, and the reduction is statistically significant (s=t=6, $P<0\ 05$) both for the initial force and the shift of the dose response curve to NA The mean reduction in initial contractile force produced by $2\ 5\times10^{-5}$ M thioridazine (26 3 %) was smaller than that produced by the same concentration of promazine, but the reduction is highly significant statistically, as is the shift of the dose-response curve for NA (s=t=6, $P<0\ 005$)

Rate of contraction

Noradrenaline increased the rate of contraction in the spontaneously beating atria, and a dose-response curve could be worked out for this effect (fig 2)

Promazine in concentrations of $2\ 5\times10^{-6}$, 5×10^{-6} and 10^{-5} M altered the shape of the dose-response curve (fig 2) in the same way as the drug altered the shape of the curve for contractile force The lowest concentration used ($2\ 5\times10^{-6}$ M) increased the rate of contraction (mean increase=19 3 %, s=8, t=6, $P<0\ 05$), and the rate after exposure to 2×10^{-8} and 10^{-7} M NA was significantly higher than in the controls (s=8, t=6, $P<0\ 001$ and $P<0\ 05$ respectively) The rate of contraction also appeared to be slightly increased after exposure to 5×10^{-6} M promazine, but this difference was not statistically significant, nor was the apparent reduction of the response to 10^{-5} M NA

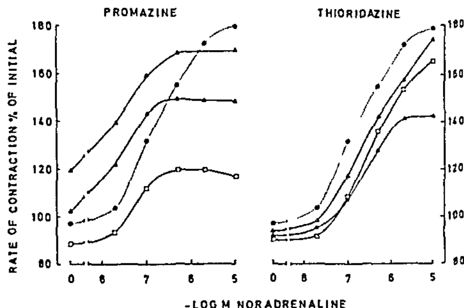


Fig 2 Log dose response curves for the effect of NA on the rate of contraction in spontaneously beating rat atria treated with promazine or thioridazine ● control △— 2.5×10^{-6} M ▲— 5×10^{-6} M, □— 10^{-5} M Each curve represents the mean of 6–8 experiments For statistical evaluation see text

Promazine 10^{-5} M on the other hand, gave a significant ($s=t=8$, $P < 0\ 05$) reduction in the rate of concentration and as a result the entire dose response curve to NA was shifted downwards ($P < 0\ 07$ for NA 2×10^{-8}, $P > 0\ 1$ for 10^{-7}, $P < 0\ 005$ for 5×10^{-7}, 2×10^{-6} and 10^{-5})

Thioridazine (fig 2) had no definite effect on the rate of contraction in the concentrations used ($2\ 5 \times 10^{-6}$, 5×10^{-6} and 10^{-5} M) and although the dose-response curve for NA lies below the control in the presence of any of the concentrations of thioridazine used the difference in rate is statistically significant ($s=t=8$, $P < 0\ 05$) only for 10^{-5} M NA in the presence of 5×10^{-6} M thioridazine Thioridazine therefore appears to have far less effect on the rate of contraction than on contractile force

Work index

Noradrenaline naturally increased the work index (=number of beats per minute multiplied by contractile force developed in each beat, LOEB 1965) since it increased both the rate and force of contraction Fig 3 shows dose-response curves for the effect of NA on the work index in spontaneously beating atria

Promazine had no apparent direct effect on the work index The increase in rate in response to $2\ 5 \times 10^{-6}$ M was counteracted by a reduction in con

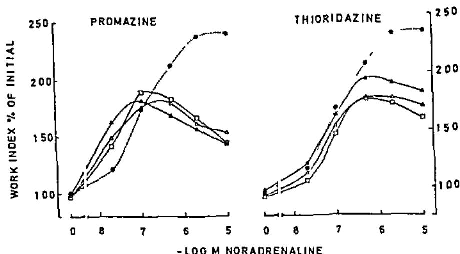


Fig 3 Log dose response curves for the effect of NA on the work index in rat atria treated with promazine or thioridazine ● - control, △—2.5×10^{-6} M, ▲—5×10^{-6} M, □—10^{-5} M Each curve represents the mean of 8 experiments For statistical evaluation see text.

tractile force, and the reduction in rate in response to 10^{-5} M was associated with a compensatory increase in contractile force These changes correspond to the "negative staircase" effect which is observed in rat atria when the rate of contraction is changed by electrical stimulation (LANDMARK 1972b) The increase in work index after exposure to 2×10^{-8} and 10^{-7} M NA was augmented by all the concentrations of promazine used – but the difference in the dose-response curves are statistically significant only for NA 2×10^{-8} M in the presence of 2.5×10^{-6} ($s=8$, $t=6$, $P<0.01$) and 5×10^{-6} M ($s=t=8$, $P<0.01$) promazine All concentrations of promazine markedly reduced the response to the highest concentrations of NA (see fig 3) ($P<0.01$ for both NA 2×10^{-6} and 10^{-5} M at promazine 2.5×10^{-6}, 5×10^{-6} and 10^{-5} M)

Thioridazine (fig 3) had no apparent direct effect on the work index in concentrations of 2.5×10^{-6}, 5×10^{-6} and 10^{-5} M, the slight reduction in rate was as a rule compensated for by a slight increase in force The responses to the highest concentrations of NA on the other hand, were significantly reduced ($s=t=8$, $P<0.02$ for both NA 2×10^{-6} and 10^{-5} M with thioridazine 2.5×10^{-6}, 5×10^{-6} and 10^{-5} M)

Discussion

The present study shows that promazine and thioridazine can depress atrial rate and force of contraction, and that the magnitude of the effect depends both on the drug used and the concentration This is in accordance with

earlier findings (LANDMARK 1971a) and is probably due to the membrane stabilizing effects of the drugs But the present investigation also shows that under certain circumstances the phenothiazines can augment atrial contractile rate and force and that the effects of promazine and thioridazine differ slightly

The lowest concentrations of promazine used caused an increase both in contractile force (promazine 5×10^{-6} M) and in the rate of contraction (promazine $2\ 5 \times 10^{-6}$ M) while no corresponding increase was obtained with thioridazine The cause of this stimulatory effect of promazine is unknown It has been shown that acetylcholine is released from post ganglionic cholinergic fibres when suprathreshold stimulation is used (VINCENZI & WEST 1965 RUBIN 1970 LANDMARK & REFSUM unpublished results) As promazine and thioridazine have anticholinergic activity (HAFFNER & LANDMARK 1972) the direct stimulatory effect of promazine in the electrically stimulated preparation could be due to an antagonism to the cardio-depressive effect of liberated acetylcholine This would explain why an increase in contractile force was observed in the electrically stimulated preparations and not in the spontaneously beating preparation where the amount of liberated acetylcholine is far smaller But it does not explain why the rate of contraction increased in the spontaneously active preparations Furthermore if the stimulatory effects of promazine were due to the anticholinergic activity thioridazine would be expected to give a similar effect since this drug also has anticholinergic properties

Several investigators have reported on the anti adrenergic effect of chlorpromazine and other phenothiazines (COURVOISIER *et al* 1953 MARTIN *et al* 1960 MADAN 1963, GOKHALE *et al* 1964 THOENEN *et al* 1965 WEBSTER 1965) These reports indicate that the phenothiazines competitively inhibit responses which are mediated by adrenergic α receptors while they appear to potentiate the adrenergic β receptor response

The cardio-stimulatory effects of NA are generally attributed to stimulation of adrenergic β-receptors and it would therefore be expected that the response to NA should be augmented by promazine and thioridazine This was also found to be the case in the present investigation but only to a limited extent Promazine augmented the response to low concentrations of NA ($< 10^{-7}$ M) but the effects of higher concentrations of NA were unaltered or reduced It has been reported that low concentrations of chlorpromazine reduce the uptake of NA into sympathetic nerve endings and thus potentiates the effect of exogenously applied NA (THOENEN *et al* 1965) The same mechanism could explain why promazine augments the effect of low concentrations of NA The direct cardio stimulatory effect of low concentrations of promazine could also conceivably be due to a reduction in the neuronal uptake of spontaneously released NA The lack of augmentation at high con

centrations of NA may be due to a reduced importance of neuronal uptake when excess NA is present

Whatever the mechanism of action, the findings recorded above may have some clinical value First of all they have shown that low concentrations of the phenothiazines may increase cardiac activity, and especially when the heart is adrenergically stimulated As the phenothiazines are used in patients who are generally in a state of anxiety, and presumably have raised blood levels of adrenaline this might be of practical importance Secondly the results show that the cardio stimulatory effects of promazine is far more marked than those of thioridazine This should be taken into account when choosing between the two phenothiazines

Acknowledgements

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REFERENCES

Courvoisier S J Fournel R Ducrot M Kolsky & P Koetschet Propriétes pharmacodynamiques du chlorhydrate de chloro 3 (diméthylamino-3 propyl 10 phénothiazine (4560 R P) *Arch int Pharmacodyn* 1963 92 305 361

Gokhale S D O D Gulati & H M Parikh An investigation of the adrenergic blocking action of chlorpromazine *Brit J Pharmacol* 1964 23 508–520

Haffner J F W & K Landmark Opposing anticholinerg c and cardiodepressive effects of promazine and thioridazine in isolated rat atria *Acta pharmacol et toxicol* 1972 31 529 539

Landmark K The action of promaz ne and thioridazine in isolated rat atria 1 Effects on automaticity mechanical performance refractoriness and excitability *Eur J Pharmacol* 1971a 16 1 7

Landmark K Changes in rat atrial action potentials induced by promazine and [illegible]

Lanc [illegible]

Land [illegible] st mulat on on contractile force and excitability *Eur J Pharmacol* 1972b 17 365–374

Landmark K J F W Haffner & J Setekleiv Reduction in ^{4}K efflux from rat atria by promazine and thioridazine *Acta pharmacol et toxicol* 1972 31 54–64

Loeb T *Comparative evaluation of methods used for the measurements of the effects of drugs on card ac muscle* M Sc Thesis University of Sidney 1965

Madan B R Some card ovascular actions of thioridazine hydrochloride *Arch int Pharmacodyn* 1963 144 299 309

Martin W R J L Riehl & K R Unna Chlorpromazine III The effects of chlorpromazine and chlorpromazine sulfoxide on vascular responses to 1-epinephrine and levarterenol *J Pharmacol exp Therap* 1960 130 37–45

Rubin R P The role of calcium in the release of neurotransmittor substances and hormones *Pharmacol Rev* 1970 22 389–428

Thoenen H A Hürlimann & W Haefely On the mode of action of chlorpromazine on peripheral adrenergic mechanisms *Int J Neuropharmacol* 1965 4 79–89

Vincenzi F F & T C West Modification by calcium of the release of autonomic mediators in the isolated sinoatrial node *J Pharmacol exp Therap* 1965 150 349–360

Webster R A The antiadrenaline activity of some phenothiazine derivatives *Brit J Pharmacol* 1965 25 566–576

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Lack of Effect of Pinealectomy on the Diurnal Rhythm in Drug Metabolism

By

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Keywords Pinealectomy – diurnal rhythm – drug metabolism

Daily rhythmic variations in the *in vitro* metabolism of drugs by rat liver were first reported by RADZIALOWSKI & BOUSQUET (1967) This finding has been confirmed by another group of investigators (JORI *et al* 1971), who demonstrated that the minimum and maximum of the drug metabolism is dependent on the schedule of the illumination conditions WURTMAN & AXELROD (1965) found in the pineal gland diurnal rhythm which was related to the natural 24 hour cycle in photic input Because of the rhythmic change in the activity of pineal function it has been suggested that the mammalian pineal gland might function as a kind of 'biological clock', which delivers time signals generated by light and darkness to centres in the brain which mediate and synchronize other biological rhythms

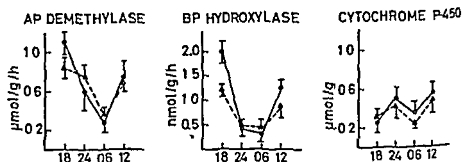
Pinealectomy has been found to level off to some extent the diurnal variation in the motility of rats (AIRAKSINEN & KARPPANEN 1970) Keeping in mind the diurnal rhythm of drug metabolism and the effect of illumination on this we have studied the effect of pinealectomy on the metabolism of 3,4 benzpyrene and aminopyrine and the concentration of cytochrome P-450 at different times of the day The plasma concentration of corticosterone was also determined

Male Sprague-Dawley rats weighing 200–300 grams were used for the experiment For pinealectomy rats were anaesthetized by sodium pentobarbital (mebumalum NFN) (40 mg/kg intraperitoneally) The pineal gland was destroyed by a stereotactic instrument according to the method of KARPPANEN *et al* (1970) The developer of the method performed all the operations The mortality, mostly due to the anaesthetic, was 18 % for the operated rats and 15 % for the controls The control rats were left intact and they were only anaesthetized The percentage of succesful pinealectomies by

this method was about 90 All the operated animals were used for the experiment

During the whole experiment the animals were kept in the same constant environment A 12 hour light period beginning at 8 a m and ending at 8 p m was provided One group of the animals was killed after one week and the other group four weeks after the operation The control animals were killed at the same time The microsomal pellet was separated by the standard ultra-centrifugation schedule and used for subsequent determinations comprising hydroxylation of 3,4 benzpyrene (KUNTZMAN *et al* 1966), N-demethylation of aminopyrine (COCHIN & AXELROD 1959), and cytochrome P-450 (OMURA & SATO 1964) Plasma corticosterone was estimated fluorometrically according to the method of ZENKER & BERNSTEIN (1958)

The results are summarized in the figure The metabolism was at the lowest level at 6 a m and at the highest level at 6 p m and the difference between *the minimum and maximum was marked in most cases Both 1 and 4 weeks*



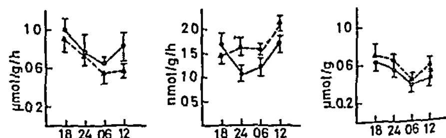


Fig 1 The effect of pinealectomy on drug metabolism 1 and 4 weeks after pinealectomy in the rat
Aminopyrine (AP) demethylase 3 4 benzpyrene (BP) hydroxylase and cytochrome P-450 were determined from the liver microsomal fraction O——O control rats Δ - - - Δ pinealectomized rats Time points are means (± standard errors) from 4 animals in the 1 week group and 5–6 animals in the 4-week group

after pinealectomy the diurnal rhythm in drug metabolism was detectable and there were no clear differences between the activities of the control and pinealectomized animals We have no clear explanation for some of the exceptions from the over all rhythmic daily variation in drug metabolism. One week after the operation the diurnal rhythm in the plasma corticosterone level was less marked in the pinealectomized than in the control animals This levelling off, however, was not significant and it was no longer observable at four weeks In agreement with this NIR *et al* (1971) reported that by using about similar light schedules as in this study, pinealectomy did not cause any significant change in the plasma corticosterone level, although a tendency to increased values was seen

Our results confirm previous findings with regard to the existence of a diurnal rhythm in drug metabolism Our observations about the activities of drug metabolizing enzymes agree rather well with the recent results of JORI *et al* (1971) published during the preparation of this study They also stated that the change in drug metabolism corresponds to a change in the plasma corticosterone level, as shown in the present study The lack of significant changes in diurnal rhythm after pinealectomy seems to suggest control mechanisms other than that associated with the pineal gland or that the pineal gland is not a crucial organ in the diurnal control of drug metabolism

Acknowledgements

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REFERENCES

Airaksinen M M & H Karppanen Effect of pinealectomy and oxypertine on hypothalamic FSH releasing factor and circadian activity rhythm in rats *Scand J Clin Lab Invest* 1970 25 Suppl 113 43

Cochin J & J Axelrod Biochemical and pharmacological changes in the rat following chronic administration of morphine nalorphine and normorphine *J Pharmacol exp Therap* 1959 **125** 105 110

Jori A E Di Salle & V Santini Daily rhythmic variation and liver drug metabolism in rats *Biochem Pharmacol* 1971 20 2965 2969

Karppanen H H Vapaatalo S Lahovaara & M K. Paasonen Studies with pinealectomized rats *Pharmacology* 1970 3 76–84

Kuntzman R L C Mark, L Brand W Levin & A H Conney Metabolism of drugs and carcinogens by human liver enzymes *J Pharmacol exp Therap* 1966 **152** 151 156

Nir I U Schmidt N Hirschmann & F G Sulman The effect of pinealectomy on rat plasma corticosterone levels under various conditions of light *Life Sci* 1971 10 317 324

Omura, T & R Sato The carbon monoxide binding pigment of liver microsomes *J Biol Chem* 1964 239 2370–2378

Radzialowski, F M & W F Bousquet Circadian rhythm in hepatic drug metabolizing activity in the rat *Life Sci* 1967, 6 2545-2548
Wurtman, R J & J Axelrod The pineal gland *Sci Amer* 1956, 213 50-60
Zenker, N & L. E. Bernstein Original method for determination of plasma corticosterone *J Biol Chem* 1958, 231, 695-701

Acta pharmacol et toxicol 1973, 32, 161–178

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Mental and Psychomotor Effects of Diazepam and Ethanol

By

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Abstract The performance of eight healthy young males was tested by means of 8 psychological and psychomotor tests 1¾ and 4½ hrs after the administration of either ethanol, diazepam 10 or 20 mg or placebo The mean plasma concentrations of diazepam were estimated to be 230 (10 mg dose) and 495 ng/ml (20 mg) respectively after 1¾ hr and 145 (10 mg), and 310 ng/ml (20 mg) after 4½ hrs, ethanol was estimated to be 0 091% after 1¾ hr and 0 049% after 4½ hrs Diazepam produced stronger subjective sensations of relaxation and reduced concentration than ethanol, whereas the test subjects experienced a similar depressing effect on motivation and effectivity after ethanol and after the largest dose of diazepam (20 mg) Both diazepam and ethanol reduced the scores in the following tests Memorizing Sorting (of coloured tablets) Complex Coordination and Critical Flicker Fusion Frequency The scores in Letter Cancellation and Mirror Tracing tests were also reduced by both drugs, but in different ways, ethanol made the test subjects attempt more but increased their number of errors, while diazepam on the other hand slowed the subjects so that they made fewer mistakes, but attempted less Diazepam (20 mg) also reduced their time evaluation ability, while ethanol did not affect it significantly Judged by clinical examination diazepam had a less marked effect on proprioception speech and balance than ethanol The effect of diazepam on the Flicker Fusion and Mirror Tracing tests could still be recognized 4½ hr after administration of the drug

Key words Diazepam – ethanol – psychomotor performance – traffic safety

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In recent years the consumption of sedatives and tranquilizers has increased considerably These drugs are not only used in psychiatry, but also as hypnotics and for less well defined indications by people who wish to alleviate the stresses of modern life Sedatives and tranquilizers are also used as premedication for minor surgery in outpatient treatment

It is well known that larger doses of diazepam – one of the most commonly used tranquilizers – may produce intoxication and impairment of mental and

psychomotor functions. Such impairment may be of great practical and legal importance in certain situations, e g. driving motor cars or flying aircraft It is therefore of interest to study the effects of varying doses of diazepam, and to compare them with the effects of other well known intoxicants, as for example alcohol.

The aim of the present study was to determine whether and to what extent a single, large therapeutic dose of diazepam affects mental and psychomotor functions in man The effects of diazepam in doses of 10 and 20 mg/70 kg body weight have been compared with those of alcohol in amounts necessary to produce blood levels of approximately 0.1 %. The serum concentrations of diazepam were estimated in order to see whether it was possible to establish a correlation between doses, serum concentrations and effects

Materials and Methods

Test subjects The investigation was carried out on 8 healthy male student volunteers, weighing from 62 to 80 kg and aged between 24 and 29 years Only one of the test subjects had tried diazepam previously, more than one month previous to the present investigation, while the others had never been exposed to the drug None of the test subjects used any drug regularly, and their alcohol consumption was moderate

Before the investigations on the drug effects were started, the students were allowed to familiarize themselves with the tests They were allowed an equal number of practice trials of the tests which were known to improve with practice, in order to minimize the effect of learning

Drugs used Diazepam was administered as tablets of 2 mg which were swallowed with water Both the diazepam and the placebo tablets were kindly supplied by A/S Apotekernes Laboratorium for Specialpreparater, Oslo, specially for the present investigation The alcohol given was administered as a mixture of 96 % v/v ethanol and fruit juice, in a final concentration of approximately 30 % v/v

Experimental design Each subject was tested on 4 different test days at intervals of 14 days, after the administration of either 10 mg diazepam per 70 kg body weight, 20 mg diazepam per 70 kg body weight, 1 22 ml 96 % ethanol/kg body weight or placebo tablets The order in which the subjects received placebo or drugs was randomized and arranged as shown in table 1 Neither the test subjects nor those who recorded the test results were told what amount (0, 10 or 20 mg) of diazepam the test subjects received

Test procedure The drugs were given in the morning after total fasting for 10 hours One hour after drug administration the subjects were allowed to eat 4 half scones and drink 2 glasses of water

Twenty minutes later the tests started with a common session consisting of 10 min "Cancellation Test", 5 min "Modified Osgood Test" and 5 min "Sorting Test".

Venous blood samples were then taken (1 hr 45 min after drug administration) for the determination of both diazepam and alcohol

After this the subjects were sent to four different "stations" where each of them spent 15 min before circulating to another station The tests carried out at these stations were 1 Flicker Fusion Frequency and Time Evaluation Ability, 2 Complex Coordination Test, 3 Mirror Tracing Test (At station 3 the subjects also went through

Table 1.

Experimental design

Test person no		1	2	3	4	5	6	7	8
Test day	1	P	A	10	20	20	10	A	P
	2	A	10	20	P	10	A	P	20
	3	10	20	P	A	A	P	20	10
	4	20	P	A	10	P	20	10	A

P=placebo 10=10 mg diazepam
A=ethanol 20=20 mg diazepam

a psychophysiological test with determination of Galvanic Skin Resistance and Heart Rate Description and results of these tests will be published separately) 4 Clinical Examination

The order in which each subject took the tests was the same on each test day The entire testing procedure was completed within 3 hrs of the administration of the drugs

The subjects were then allowed one hour's rest, sandwiches and one cup of coffee, after which the entire procedure was repeated with a new blood sample taken at 4 hrs 30 min after the administration of the drugs

Description of tests

Modified Osgood Test The subjects graded their own mental state by choosing one of seven stages (−3, −2 −1, 0, 1, 2, 3) on the following ranges 1 Tense – relaxed, 2 Excited – at ease, 3 Unpleasant – pleasant 4 Unmotivated motivated, 5 Unconcentrated – concentrated, 6 Inefficient – efficient, and 7 Inattentive – attentive For a thorough discussion of this kind of measurements, see OSGOOD *et al* 1957

Time Evaluation Ability This was determined simply by stating when a stop-clock was started, and also when it was stopped The time as assessed by the subject was compared with the actual time Five time intervals varying between 5 and 65 sec were used, and the difference between the sums of the 5 actual times and the 5 assessed times was determined

Letter Cancellation Test The subjects cancelled out letters in a continuous text of nonsense – syllables of varying lengths applying the three following rules of cancellation simultaneously 1 Single letters between two wovels (Example bu ⁄a ypt) 2. The last of any two identical letters standing next to each other (disregarding the distance between the letters) (Example ap⁄p ke ⁄e) 3 Any pair of letters succeeding another pair (Example xy oh p⁄y) The numbers of skipped and correct cancellations were noted

Sorting Test The subjects placed wooden tablets of four different colours into nine different holes which were arranged in three rows Each hole was marked with the printed name of a colour and also had a coloured brim (as a rule in a different colour) The tablets were sorted according to orders given at increasing speed by tape recorder The orders contained *colour* (of tablets) *1 2 or 3 knocks* (for the row of holes) and *either colour* (coloured brim) or *initial letter of colour* (printed name) The difference between correctly and wrongly placed tablets was used as test score

The Time Evaluation Ability, Letter Cancellation and Sorting Test have been developed in The Psychological Service of The Norwegian Armed Forces All the tests are used for personnel selection and classification Letter Cancellation has proved to be a

good substitute for the somewhat "clumsier" Complex Coordination test and gives much of the same information The Sorting Test has proved valuable as a test of the ability to perform under stress (See Technical Report The Psychological Service of The Norwegian Armed Forces 1961-71)

Flicker Fusion Frequency (FFF) Flicker was obtained by interrupting a beam of light with a rotating disc from which two 90° sections had been cut out The intervals of light and dark were therefore equal The light was projected on to a frosted glass which was viewed by the test object from a fixed position 80 cm away The brightness of the frosted glass was 2000 Lux when light was continuous and the metal plate surrounding the glass was lit by background lighting to a brigthness of 22 Lux The intensity of the light was kept constant, while the flicker frequency was increased stepwise from a frequency of 40 flicks second $^{-1}$, in steps of 1 or 2 flicks second $^{-1}$ The test subject was instructed to indicate verbally when the light *stopped flickering*, this frequency of flicker was denoted as FFF The subjects were allowed to view the frosted glass for approximately 1 sec at each step, in between exposures the glass was covered manually by a white cardboard plate At each session FFF was determined six successive times and the mean was calculated from these data

The test was thoroughly reviewed in 1952 by SIMONSON & BROZEK, *alterations in FFF* are believed to indicate alterations in cortical functions

Complex Coordination Test This test was used for pilot selection in World War II The subject is told to imitate a pattern of three lights displayed in front of him by moving three other lights The lights are moved by means of a joystick which controls one light in a vertical and one in a horizontal row, and by a foot bar which regulates one light in another horizontal row When the light pattern has been correctly imitated and held for half a second it is automatically switched off, and a new pattern appears The number of correct imitations achieved within 9½ min was recorded

Mirror Tracing Test This test had much in common with WASKOM s (1936) The subject s task is to move a pencil shaped brass stylus along a star shaped track in a brass plate (without lifting the stylus, pressing it too hard touching the sides of the track, or touching the brass plate with his hand Each time any of these errors is made a buzzer sounds The stylus and the track are shielded from vision and can only be viewed through a mirror In the present experiments the following parameters were scored A Total time required to complete the track B Number of times the sides of the track was touched (number of errors) C Total time spent on correction of error

Clinical Examination was carried out according to the method used by Norwegian Police Surgeons to test drunken drivers The clinical tests were graded as *affected* (+) or unaffected (0) The seven most sensitive tests which were scored (table 8) were 1 *Gait* Walking along a straight line and turning about when ordered 2 *Turning* around on the spot 3 *Balance* Standing on one leg with closed eyes 4 *Proprioception* Finger-to-nose and finger-to-finger with closed eyes 5 *Speech* Reading from a standardized text which included some difficult words 6 *Concentration* The ability to count backwards from a number between 100 and 150 7 *Memorizing* an address and a phone number read out before the speech and concentration tests

Presentation of results The mean of the scores for the eight test subjects is given in tables 2–8, where the effects of the different treatments are compared In addition the differences between the mean scores after placebo and drugs are given The statistical significance of these mean differences was evaluated by Wilcoxons test for paired comparisons

The concentration of alcohol in blood plasma was determined by the ADH method

The determination of diazepam in plasma was carried out by gas chromatography according to a modification of the method described by BERLIN *et al* (1972) The method is based on extraction of diazepam with benzene from plasma mixed with equal amount of saturated KCl solution The benzene solution is evaporated almost to dryness under nitrogen The residue is dissolved in 100 μl benzene containing 100 picogram griseofulvin per microliter as internal standard An amount of 5 μl of this solution is injected into the gas chromatograph

A Varian Aerograph model 1400 equipped with a ^{63}Ni detector was used The silanized glass column (6 ft × 3 mm i d) was packed with 3 % OV – 17 on 80/100 mesh Gas Chrom Q The temperatures were 305° in the injector, 265° in the column and 310° in the detector The flow rate of the nitrogen was 45 ml per min

The concentrations in the plasma were calculated from the peak heights using standard curves prepared by subjecting plasma with known concentrations of diazepam to the same procedure

Results

Modified Osgood Test Figs 1 and 2 show diagramatic presentations of the results of this test One hr 30 min after the administration of 10 mg diazepam the test subjects felt moderately relaxed, at ease, pleasant, unconcentrated, inefficient and inattentive as compared to the condition after placebo Diazepam 20 mg also increased the feeling of relaxation and ease, reduced motivation and made the subjects less concentrated, less efficient and less attentive Alcohol caused some relaxation, but otherwise had negative effects

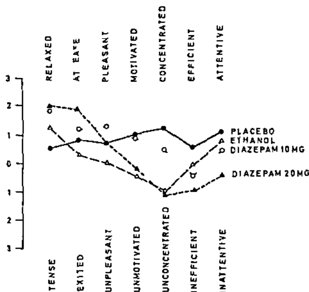


Fig 1 *Modified Osgood Test* Mean of the mental stages indicated by the 8 subjects Time after drug administration 1 hr 30 min

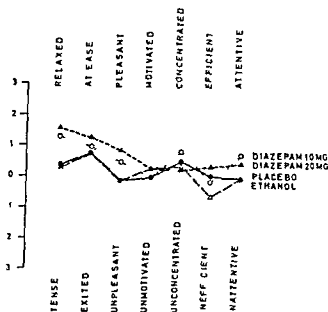


Fig 2 *As fig 1* Time after drug administration 4 hrs 10 min

Four hours after drug administration the mental state deviated less from that after placebo

Time Evaluation Ability The mean value of the sum of the assessed times after placebo was 126 sec i e 24 sec shorter than the actual time (150 sec) in the first test (2 hrs 20 min) In the second test (5 hrs) the assessed time was 27 sec shorter than the actual time (table 2) The only statistically significant result was recorded after 20 mg diazepam in the first test, when the assessed time was 25 sec shorter than after placebo

Letter Cancellation Test Table 3 A shows that in the first test (1 hr 30 min) alcohol administration was followed by a significant increase in the number of cancellations attempted while after 20 mg diazepam there was a significant reduction of such attempts The number of skipped cancellations increased after alcohol, but decreased after 20 mg diazepam both in the first (table 3B) and second test (4 hrs 10 min) The reduction in the number of correct cancellations was statistically significant only for 20 mg diazepam in the first test (table 3 C)

The number of skipped and correct cancellations was also converted into percentages of the number of attempted cancellations in order to see whether the relative proportions had changed Alcohol markedly increased the percentage skipped (table 3 B) and reduced the percentage of correct cancellations (table 3 C) while diazepam had no significant effect on these percentages

Table 2

Time Evaluation Ability

Test period (mean time after drug)	1 (2 hrs 20 min)				2 (5 hrs)			
Treatment	Placebo	Diazepam 10 mg	Diazepam, 20 mg	Ethanol	Placebo	Diazepam, 10 mg	Diazepam, 20 mg	Ethanol
Real time-assessed time (sec.)	24.2	38.3	50.0	32.9	27.3	31.0	33.6	18.1
Placebo-Test (sec.)	–	–14.1*	–25.8	–8.7	–	–3.7*	–6.3	9.2
Significance (Placebo-Test)	–	– *	< 0.1	> 0.1	–	– *	> 0.1	> 0.1

*) Results from only 7 subjects, as one subject estimated the time 206 sec too long in the first period and 121 sec too long in the second period

Table 3.

Letter-Cancellation Test.

Test period (mean time after drug)	1 (1 hr 30 min)				2. (4 hrs 10 min)			
Treatment	Placebo	Diazepam, *10 mg*	Diazepam, *20 mg*	Ethanol	Placebo	Diazepam, 10 mg	Diazepam, 20 mg	Ethanol
A Attempted cancellations								
Mean number	173.0	159.4	142.6	200.4	172.8	183.5	170.6	180.6
Placebo-Test	–	13.6	30.4	–27.4	–	–10.8	2.2	–7.6
Significance (Placebo-Test)	–	> 0.1	< 0.05	< 0.02	–	> 0.1	> 0.1	> 0.1
B Skipped cancellations								
Mean number	45.1	37.8	32.0	77.4	39.4	37.9	34.1	44.1
In per cent of attempted cancellations	26.1	23.7	22.5	38.5	22.8	20.6	20.0	24.4
Placebo-Test	–	7.4	13.1	–31.3	–	1.5	5.3	–4.7
Significance (Placebo-Test)	–	> 0.1	< 0.01	< 0.05	–	> 0.1	0.1	> 0.1
C Correct cancellations								
Mean number	127.9	121.6	110.6	123.0	133.1	145.6	136.5	136.5
In per cent of attempted cancellations	73.8	76.3	77.5	61.4	77.2	79.6	80.0	75.6
Placebo-Test	–	6.3	17.3	4.9	–	–12.5	–3.4	–3.4
Significance (Placebo-Test)	–	> 0.1	0.1	> 0.1	–	> 0.1	> 0.1	> 0.1

Table 4.

Sorting Test

Test period (mean time after drug)	1 (1 hr 30 min)				2 (4 hrs 10 min)			
Treatment	Placebo	Diazepam, 10 mg	Diazepam, 20 mg	Ethanol	Placebo	Diazepam, 10 mg	Diazepam, 20 mg	Ethanol
Mean score	38 9	35 3	35 4	34 9	39 9	40 0	41 4	39 1
Placebo-Test	–	3 6	3.5	4 0	–	-0 1	-1.5	0 8
Significance (Placebo-Test)	–	> 0 1	< 0 02	< 0 01	–	> 0 1	> 0 1	> 0 1

Table 5

Flicker Fusion Frequency

Test period (mean time after drug)	1 (2 hrs 20 min)				2 (5 hrs)			
Treatment	Placebo	Diazepam, 10 mg	Diazepam, 20 mg	Ethanol	Placebo	Diazepam, 10 mg	Diazepam, 20 mg	Ethanol
Mean FFF (sec^{-1})	52 1	50 4	50 1	50 1	52 0	51 0	50.8	51 9
Placebo-Test (sec^{-1})	–	1 7	2 0	2 0	–	1 0	1 2	0 1
Significance (Placebo-Test)	–	< 0 01	< 0 01	< 0 02	–	< 0 05	< 0 05	> 0 1

Table 6

Complex Coordination Test

Test period (mean time after drug)	1 (2 hrs 20 min)				2 (5 hrs)			
Treatment	Placebo	Diazepam, 10 mg	Diazepam, 20 mg	Ethanol	Placebo	Diazepam, 10 mg	Diazepam, 20 mg	Ethanol
Mean score	110 4	108 8	102 6	91 4	118 6	115 6	114 4	109 0
Placebo-Test	–	1 6	7 8	19 0	–	3 0	4 2	9 6
Significance (Placebo-Test)	–	> 0 1	< 0 05	< 0 01	–	> 0 1	0 1	< 0 1

Table 7

Mirror Tracing Test

Test period (mean time after drug) Treatment	1 (2 hrs 20 min) Placebo	Diazepam 10 mg	Diazepam, 20 mg	Ethanol	2 (5 hrs) Placebo	Diazepam, 10 mg	Diazepam, 20 mg	Ethanol
A Total time								
Mean time (sec)	126.1	160.7	177.7	134.8	128.8	141.4	147.9	122.0
Placebo-Test (sec)	–	–34.6	–51.6	–8.7	–	12.6	–19.1	6.8
Significance (Placebo-Test)		< 0.05	< 0.01	> 0.1	–	> 0.1	< 0.05	> 0.1
B Errors								
Mean number	53.6	63.4	71.0	100.5	52.0	46.4	54.4	49.4
Placebo-Test	–	–9.8	–17.4	–46.9	–	5.6	–2.4	2.6
Significance (Placebo-Test)	–	> 0.1	0.1	< 0.02	–	> 0.1	> 0.1	> 0.1
C Time spent on correction of error								
Mean total time (sec)	9.75	12.78	16.56	19.57	9.61	8.60	10.53	8.88
Placebo-Test (sec)	–	–3.03	–6.81	–9.81	–	1.01	–0.92	0.73
Significance (Placebo-Test)		> 0.1	< 0.05	< 0.05	–	> 0.1	> 0.1	> 0.1
D Number of errors divided by total time (sec.$^{-1}$)	0.43	0.39	0.40	0.75	0.40	0.33	0.37	0.41
E Total time spent on correction of errors divided by Number of errors (sec)	0.18	0.20	0.23*	0.19	0.18	0.19	0.19	0.18

* Significance Placebo-Test < 0.02

Table 8

Clinical Examination

Test period (mean time after drug)	1 (2 hrs 20 min)				2. (5 hrs)			
Treatment	Placebo	Diazepam, 10 mg	Diazepam, 20 mg	Ethanol	Placebo	Diazepam, 10 mg	Diazepam, 20 mg	Ethanol
1 Gait (+)*	0	0	0	0	0	0	0	0
2 Turning (+)*	0	0	1	1	0	0	0	1
3 Balance (+)*	0	1	3	6	0	0	0	1
4 Proprioception (+)*	0	1	0	4	1	0	1	1
5 Speech (+)*	0	1	1	5	0	0	1	3
6 Concentration (+)*	0	1	2	3	1	0	1	0
7 Memorizing (+)*	1	4	5	5	5	5	6	4
Sum (+)	1	8	12	24	7	5	9	10
Mean	0.13	1.0	1.5	3.0	0.87	0.63	1.13	1.25
Placebo-Test (+)	–	–0.87	–1.37	–2.87	–	0.24	–0.26	–0.38
Significance (Placebo-Test)	–	< 0.05	< 0.01	< 0.01	–	> 0.1	> 0.1	> 0.1

* The figures given indicate the total number of test subjects registered as affected (+)

Sorting Test The score at 1 hr 30 min was reduced both by 20 mg diazepam and alcohol, the latter having the greater effect Ten mg of diazepam also reduced the score, but the effect was not statistically significant (table 4) Neither diazepam nor ethanol influenced the scores in the second test (4 hrs 10 min)

Flicker Fusion Frequency After the drugs there was a reduction in the ability to recognize flicker, i e the subjects indicated that the flicker disappeared at a lower frequency (table 5) In the second test (5 hrs after drug) alcohol no longer reduced the FFF, while both doses of diazepam still produced this effect

Complex Coordination Test Both alcohol and 20 mg diazepam reduced the number of correct imitations achieved after 2 hrs 20 min Diazepam 10 mg had no significant effect After 5 hrs both diazepam and alcohol reduced the score, but the effect was statistically significant only for alcohol (table 6)

Mirror Tracing Test The time required to complete this test increased after both alcohol and diazepam, although only significantly after the latter (table 7A) Even 5 hrs after 20 mg diazepam there was a significant increase in the time spent on this test. The number of errors are given in table 7B The highest number of errors in the first test (2 hrs 20 min.) occurred after alcohol, but administration of diazepam also increased the number of errors In the second test (5 hrs) neither drug increased the number of errors Table 7C shows that the total time spent on errors was greatest after alcohol, fol-

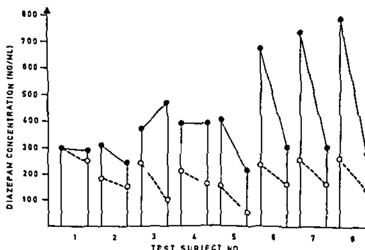


Fig 3 *Plasma diazepam concentrations* in 8 test subjects given 10 (○----○) or 20 (●——●) mg diazepam To the left of each column the values obtained 1 hr 45 min after drug administration to the right the values at 4 hrs 30 min

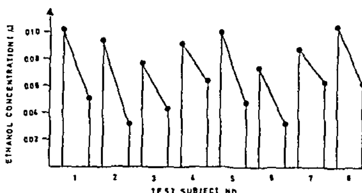


Fig 4 *Plasma ethanol concentrations* (w/w) in 8 test subjects 1 hr 45 min (highest values) and 4 hrs 30 min (lowest values) after administration

lowed by 20 mg and 10 mg of diazepam Statistically significant differences were obtained only in the first test (2 hrs 20 min.) Table 7D shows the number of errors divided by the time used to complete the test In the first test after alcohol this factor is 0 75 sec^{-1}, i e the subjects made on an average 3 errors in 4 seconds during the whole test After diazepam or placebo they made 2 errors in 5 seconds (factor=0 4 sec^{-1}) during the test The time spent on each error (table 7E) was 0 18–0 19 sec after placebo and alcohol in both test periods and after diazepam in the last period In the first period this time was 0 20 sec after 10 mg and 0 23 sec after 20 mg of *diazepam* the last figure, 0 23 sec being significantly different from placebo Thus alcohol mainly increased the frequency of errors while diazepam increased the time spent on the correction of each error

Clinical examination It was thought of interest to compare the results obtained above with the results obtained *in a clinical examination* The results are given in table 8 Alcohol was followed by marked alterations after 2 hrs 20 min Diazepam also gave a statistically significant increase in the number of positive tests recorded Five hours after drug and placebo the subjects were obviously tired and at this time no significant differences from the placebo group could be recorded *Difficulties in memorizing* accounted for about 40 per cent of the positive tests obtained on clinical examination

Plasma concentrations of diazepam and ethanol

One hour 45 min after the administration of 10 and 20 mg diazepam the mean plasma concentrations were 230 and 495 ng/ml respectively Four hours 30 min after administration the corresponding values were 145 and 310 ng/ml As shown in fig 3 the change in plasma level from the first to the second determination was quite different from subject to subject The most rapid reduction in the serum level was found in the subjects who initially

(1 hr 45 min) had the highest plasma levels (fig 3) These inter individual differences may either be due to differences in absorption or metabolism or both

Ethanol administration gave mean plasma levels of 0 091 % at 1 hr 45 min and 0 049 % at 4 hrs 30 min. respectively (fig 4) The inter individual variations were smaller than after diazepam and the mean rate of ethanol disappearance was calculated to be 0 015 % per hr

When the scores of each subject in each test were compared to this plasma level of diazepam at that time, no good correlation was found, possibly because only 8 subjects were tested. In this small material no more than a rough correlation between performance and diazepam concentration was found as seen in figs 1 and 2 and tables 2-8, 20 mg diazepam was always followed by more marked effects than 10 mg

Discussion

Driving a car or an aircraft involves intellectual as well as physical performances In the traffic situation it is of vital importance to evaluate and respond correctly and quickly to new, more or less complex situations, and to execute manoeuvers rapidly by coordinated movements

It is well known that alcohol impairs the driving ability The present investigation indicates that diazepam also has a negative influence on the results of a series of tests with relevance to driving performance In none of the tests was there any improvement in mean score after diazepam as compared with placebo However a comparison of the test results reveals some differences between the effects of alcohol and diazepam

Firstly the effect recorded by subjective evaluation of the mood deviates After the administration of 10 mg diazepam and especially after 20 mg diazepam, the test subjects reported that they were more relaxed at ease, pleasant and unconcentrated than after alcohol intake They experienced a similar depressing effect on motivation and effectivity after 20 mg diazepam as after ethanol These findings are in agreement with the results obtained by JÄÄTTELA *et al* (1971) who found that 10 mg diazepam made the test subjects significantly more inactive and, in addition the male subjects more euphoric Judged by clinical examination we also found that the ability to concentrate was impaired by diazepam.

Another difference in behaviour is the attitude towards the trials in the Letter Cancellation and the Mirror Tracing Test Under the influence of alcohol the subjects made more attempts and more errors the subjects who had taken diazepam worked more slowly, attempted less, but did not make more mistakes than in the placebo experiments The effect of a[illegible]ol might

be due to the well known initial reduction of inhibition with extinction of selfcriticism In our limited material diazepam does not appear to induce this effect in the same way as alcohol, but the subjects acted more slowly The slowness could either be due to the altered appreciation of the passage of time as shown by the results of the time evaluation test, or it could be due to slowness of motor performance If this can be confirmed in a larger material and in other studies it would be of importance in the evaluation of the traffic-risk represented by diazepam

The intellectual or mental capacity is reduced both by alcohol and diazepam In this respect the effect of alcohol and 20 mg diazepam appeared to be about equally effective in the memorizing test Even after 10 mg diazepam the memorizing ability is impaired, which is in agreement with the findings of JAÄTTELA *et al* (1971) in their test on repeating number series They used a dose of 8 mg diazepam, whereas HUGHES *et al* (1965), who used 6 mg diazepam and nine verbal or arithmetic tests, did not obtain any significant influence on the performance score

The sorting test involves integrated mental and intellectual functions such as alertness, memory and evaluation, in addition to psychomotor performances Diazepam 20 mg made the subjects significantly less fitted for this test, but alcohol impaired their ability even more

Regarding the effects of drugs on central nervous functions, the discrimination of Flicker Fusion Frequency (FFF) has proved a valid test (for ref see SIMONSEN & BROZEK 1952) We found that 10 mg diazepam significantly depressed the ability to discriminate flicker to about the same degree as the dose of ethanol given Similar findings have been reported by others (HOLMBERG 1971) A decrease in FFF which probably reflects a disturbance in the integrative action of the central nervous system, therefore seems to be a sensitive test for the influence of diazepam on central nervous function

In small doses diazepam is believed mainly to alter the mood, especially relieving anxiety and tension, probably by acting on structures in the central nervous system controlling the emotions However, in the doses given in the present study diazepam also affected the psychomotor performances Speech was little affected, as were gait and balance as compared with the effect of alcohol More affected were the results of the Sorting Test, the Complex Coordination Test and the Mirror Tracing Test These tests require integrated reflex activity of the central nervous system and coordinated movements of the limbs Diazepam impairs the test results, probably mainly by slowing down the activity and by increasing the time required to execute the responses

Since it has been shown in animal experiments that diazepam does not significantly affect monosynaptic reflexes and the peripheral nervous muscular transmission unless large doses are given, the action is probably on central

nervous mechanisms involving polysynaptic reflexes (HAMILTON 1967, PRZYBYLA & WANG 1967), presumably on the brain stem reticular formation

The tests used in the present investigation mimic only some of the performances involved during car or aircraft driving However, since the results of our tests indicate that the effects of diazepam (especially in high doses) are in many respects comparable with those of alcohol, it would be expected that both driving and flying ability are also reduced under the influence of diazepam. In addition, probably the most dangerous effect with regard to traffic safety is the well known sedative effect of diazepam, which might induce sleep in the driver and thereby cause accidents The test subjects in the present experiments were obviously sleepy after diazepam intake They were liable to fall asleep even under such inconvenient procedures as venepuncture No attempt was made, however, to estimate the degree of sleepiness

In the present investigation the plasma concentrations of diazepam only were estimated and none of its metabolites

The present material is small, but from the data obtained it can be concluded that great individual variations exist with regard to plasma concentrations obtained after the same dose of diazepam, and only a rough correlation was obtained between the effects on the tests and the plasma concentrations of diazepam However, the fact that the first plasma concentration measured after 10 mg diazepam and the second concentration measured after 20 mg diazepam, which is about the same, just gave significant effects on some of the tests used, and that 20 mg diazepam always gave greater effects than 10 mg indicates that a correlation exists In order to elucidate this correlation further, a finding which would be of importance in legal medicine, the need for a larger material is obvious

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REFERENCES

Berlin A B Siewers S Agurell A Hiort F Sjoqvist & S Strom Determination of bioavailability of diazepam in various formulations from steady state plasma concentration data. *Clin Pharmacol Therap* 1972, 13 733–744

Hamilton J Muscle-relaxant activity of chlordiazepoxide and diazepam *Canad J Physiol* 1967 45 191 199

Holmberg G Klorazepat *Nordisk psykiatrisk tidsskrift* 1971, 25 30–33

Hughes F W R B Forney & A B Richards Comparative effect in human subjects of chlordiazepoxide diazepam and placebo on mental and physical performance *Clin Pharmacol Therap* 1965 6, 139–145

Jaáttela, A , P Männistö, H Paatero & J Tuomisto The effects of diazepam or diphenhydramine on healthy human subjects *Psychopharmacologia* 1971, 21 202–211

Osgood, C. E , G J Suci & P H Tannenbaum *The measurement of meaning* University of Illinois Press, 1957, 342 pp

Przybyla, A C & S C Wang Studies on the locus of CNS action of diazepam *Pharmacologist* 1967, 9, 188

Simonson, E & J Brozek Flicker fusion frequency Background and application *Physiol Rev* 1952, 145, 349–378

Waskom H L An experimental analysis of incentive and forced application and their effect upon learning *J Psychol* 1936, 2 393–404

Acta pharmacol et toxicol 1973, 32, 179–192

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Inhibition of Carrageenin-Induced Rat Paw Oedema by Catecholamines and Amine-Depleting Drugs

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Abstract The anti inflammatory effects of adrenaline, isoprenaline, noradrenaline, tyramine, reserpine, guanethidine and fibrinolysin® were tested against carrageenin induced rat paw oedema All the substances significantly inhibited the oedema The inhibition by tyramine enhanced the effect of reserpine The MAO inhibitor iproniazid potentiated the effect of noradrenaline and reduced the effect of reserpine The *in vivo* effects of adrenaline, noradrenaline reserpine and fibrinolysin on parameters of the plasma kinin system were investigated Neither kininogen nor prekallikrein was influenced by any of the substances tested The effect of noradrenaline, reserpine and guanethidine on the blood serotonin level was also investigated Noradrenaline and guanethidine did not influence the serotonin level under experimental conditions which cause a significant reduction in the carrageenin induced rat paw oedema The depletion by reserpine of the serotonin stores did not correlate with the rat paw oedema inhibition values Nevertheless a slight reduction in the blood concentration of serotonin after pedal in-

stores rather than normal serotonin stores, and the application to the blood platelet serotonin seemed essential for the development of carrageenin induced rat paw oedema The fact that pretreatment with catecholamines inhibited the development of oedema might suggest that the same pathway of biochemical events is involved in the inflammatory process and in the preventive mechanism the inhibition then reflecting an acute activation and depletion of a factor later in the sequence

Key words Carrageenin – rat paw oedema – catecholamines – amine depleting drugs – kininogen – kallikrein – blood serotonin – plasminogen.

The anti inflammatory effect of catecholamines is well known, but the mechanism is uncertain SPECTOR & WILLOUGHBY (1964) concluded from their experiments that adrenaline might act as a natural anti inflammatory substance by causing a decrease in capillary permeability, while ROCHA E

SILVA (1964) put forward the hypothesis that injury may lead to the release of catecholamines which in turn activate proteases capable of depleting kininogen GOZSY & KÁTÓ (1966) have confirmed previous data on the anti-inflammatory effect of adrenergic substances, but in addition emphasize *that there is subsequently an increase in capillary permeability* The stimulation of β receptors has been suggested by BROWN *et al* (1968) as being involved in the oedema inhibition caused by sympathomimetics, while BHALLA *et al* (1970) conclude from their observations that the anti inflammatory effect of adrenaline was probably due to an action on the pituitary adrenal system

In the present work we decided to establish experimental conditions under which adrenergic substances could inhibit the development of carrageenin induced rat paw oedema We further wanted to investigate the effects on factors involved in the plasma kinin system, and also on the blood level of serotonin, of catecholamines and of amine-depleting drugs in concentrations which cause a significant inhibition of the inflammatory model

According to FEKETE & KURTI (1970) and CRUNKHORN & MEACOCK (1971) serotonin may play a role at some step in the development of oedema, and the last mentioned authors concluded that kinins also contribute to the formation of oedema Previously a correlation has been demonstrated for several substances, among others serotonin, between the effect on the plasma kinin system and the inhibiting effect on carrageenin induced rat paw oedema (BRISEID *et al* 1971) We also decided to investigate if catecholamines in anti-oedema effective concentrations would cause a reduction in plasminogen. The activation of the fibrinolytic system by adrenergic substances is described in several papers (BIGGS *et al* 1947, GENTON *et al* 1961, CHO & CHOY 1964, POHALA *et al* 1962, HOLEMANS 1965) The activation of the kallikrein-kinin system by plasmin is now well established (EISEN & VOGT 1970)

Technique

A Materials

Rats Male and female Wistar albino rats (body weight 160–230 g) were obtained from the National Institute of Public Health Oslo

Drugs Carrageenin Marine colloids Inc Springfield N J U S A *Adrenaline* Bitartrate d Adrenaline Rhône Poulenc, Paris France *Isoprenaline* Isoprenalini sulfas Ph Nord *Noradrenaline* L Nor Adrenalin Bitartrat Hoechst A G, Frankfurt (M) Germany *Iproniazid* Iproniazid phosphate Sigma Chem Co, St Louis Missouri U S A *Reserpine* Serpasil® CIBA Societé Anonyme Basel Switzerland *Tyramine* Tyramine HCl Sigma Chem Co St Louis Missouri U S A *Guanethidine* Guanethidine sulfate ismelin® CIBA Société Anonyme Basel Switzerland *Fibrinolysin (plasmin)* Fibrinolysin (Human) lyovac® Merck Sharp & Dohme West Point Pa

U S A The figures given for the doses of adrenaline, isoprenaline, noradrenaline and reserpine correspond to the amounts of free base injected The doses of the other drugs are expressed as the amount of the salt actually used

Rat plasma, rat plasma kininogen rat plasma kallikrein and rat urine kallikrein were prepared as described previously (*Briseid et al* 1970)

Reagents Bradykinin Bradykinin, Sandoz A G , Basel Switzerland, was used as the standard substance in the kinin assays *Serotonin* 5 hydroxytryptamine creatinine sulphate complex, Sigma Chem Co, St Louis, Missouri U S A *Casein* Casein Yellow, B grade Calbiochem, Los Angeles California, U S A *Urokinase* Urokinase reagent, 10 000 Plough units per ampoule Leo Pharmaceutical Products Copenhagen Denmark.

B Methods

Rat paw oedema test This test was carried out as described in a previously published paper (*Briseid et al* 1971)

Determination of factors of the kinin system Determinations of kininogen fractions total kininogen and rat plasma prekallikrein were as described previously (*Briseid et al* 1970) The kinin determinations were carried out on the isolated rat uterus as 'bracketing assays', with a standard dose ratio 3 2 Bradykinin was used as standard

Determination of serotonin in whole rat blood The method of Fuwiler et al (1970) was used A 1% solution of EDTA 2Na in saline was used as anticoagulant Blood up to 3 0 ml from each rat was collected from the inferior vena cava into a siliconized syringe containing 0.3 ml of the anticoagulant solution After gentle inversion a sample of 2 0 ml or 1 0 ml of the blood was placed in a 50 ml stoppered centrifuge tube and frozen and stored at −20° As a rule the sample was thawed on the next day and then carried through the serotonin determination procedure Fluorescence was determined in a Farrand MK 1 spectrofluorometer (activation wave length 303 mμ, fluorescent wave length 540 mμ) Glass-redistilled water was used for the preparation of the reagents and for the cleaning of all the glassware

Determination of plasminogen in rat plasma The caseinolytic method of JACOBSEN (1964) was applied with minor modifications 0 25 ml of the test sample (a mixture of equal amounts of citrated rat plasma and phosphate buffer 0 025 M, pH 7 65) was mixed with 0 25 ml of N/6 HCl, and the mixture was left at room temperature for 60 minutes It was then neutralized with 0 25 ml N/6 NaOH after which 0.25 ml phosphate buffer 0 1 M pH 7 65 was added Then 0 75 ml of casein solution 5 0 % (w/v) was added and the mixture was left at 22° for 10 minutes 0 75 ml of urokinase (183 U/ml) was then added and the plasminogen was activated for exactly 10 minutes at 22° The tube was then transferred to a water bath at 37.5° for caseinolysis After 30 minutes a sample of 1 00 ml was withdrawn and precipitated with 2.50 ml 1 69 M perchloric acid After shaking the tube was stored at 4° for at least 1 hour The precipitate was then removed by centrifugation at 17 500×g for 15 minutes The optical density in the clear supernatant was read at 275 mμ in a Hitachi 101 spectrophotometer Alternatively 2 50 ml of the supernatant was mixed with 0 75 ml 5 N NaOH to pH 12, and the optical density in the yellow solution was read at 428 mμ

Results

Experiments 1, 2, 3 and 4 of table 1 show that adrenaline isoprenaline and noradrenaline significantly inhibited the development of [illegible] in-

Table 1.

Effects on carrageenin induced rat paw oedema of various substances injected intraperitoneally

Number of rats in control and test groups 5
Intraperitoneal injections 1 0 ml/100 g rat
Paw injections 0 1 ml/rat of a 1 % (w/v) suspension of carrageenin in saline
For further details see text and Briseid *et al* (1971)

Experiment number	Intraperitoneal injection mg/kg	Time in hours before carrageenin	Per cent *increase in rat* paw volume after time in hours				Inhibition *per cent*
			1	2	3	Average1)	
1	Adrenaline 0 50	½	7	13	16	11 8 (S D = 3 2)	75
	Saline	½	17	53	69	46 4 (S D = 3.3)	
2	*Isoprenaline 5 0*	½	9	17	45	23 6 (S D = 3 4)	51
	Saline	½	21	52	71	47 8 (S D = 5 6)	
3	Noradrenaline 0.50	½	17	33	42	30 6 (S D = 13 2)	44
	Iproniazid 100	20½	24	56	75	51 6 (S D = 8.1)	5
	Iproniazid 100 and Noradrenaline 0 50	20½ and ½	15	19	19	18 2 (S D = 1 9)	67
	Saline	½	29	61	75	54 6 (S D = 7 3)	

4	Noradrenaline 0.50	½	19	23	37	26 6 (S D = 10 6)	35
	Noradrenaline 0.50	3	12	38	58	36 0 (S D = 10 2)	13
	Saline	3	18	45	61	41.2 (S D = 17.5)	
5	Reserpine 1 0	20	19	37	51	35 4 (S.D = 5 4)	25
	Tyramine 200	½	13	29	51	30 8 (S D = 5 0)	34
	Reserpine 1 0 and Tyramine 200	20 and ½	10	22	35	22 4 (S D = 7 4)	52
	Iproniazid 100 and Reserpine 1 0	26 and 20	15	46	63	41 0 (S D = 2.5)	13
	Saline	20 and ½	29	48	63	47 0 (S D = 11 6)	

1) Calculated as the mean of the average per cent increase from each of the five rats in the group

Table 2

Effects on carrageenin induced rat paw oedema of different doses of reserpine and guanethidine and of fibrinolysin (plasmin)

Number of rats in control and test groups 5
Paw injections 0 1 ml/rat of a 1 % (w/v) suspension of carrageenin in saline
Control groups not included in the table
For further details see text and Briseid *et al* (1971)

Experiment number	Intraperitoneal[1] injection mg/kg	Time in hours before carrageenin	Inhibition per cent Mean ± S D	Average inhibition per cent
1	Reserpine 0 05	24	5 4 ± 17 1	5
1	Reserpine 0 15	24	13 6 ± 14 6	2
2	Reserpine 0 15	20	−8 8 ± 24 0	
1	Reserpine 0 50	24	12.2 ± 22.2	5
2	Reserpine 0 50	20	−2 0 ± 25 4	
3	Reserpine 1 00	14½	42.2 ± 18 0	29
4	Reserpine 1 00	19½	21.2 ± 11 4	
5	Reserpine 1 00	20	24 6 ± 11 6	
2	Reserpine 1 50	20	33 4 ± 21 8	37
6	Reserpine 1 50	24	29 4 ± 18 4	
7	Reserpine 1 50	23	47 0 ± 11 2	
8	Reserpine 2 00[2]	21	45 0 ± 6 0	45
6	Guanethidine 10	24	5 0 ± 13 2	5
9	Guanethidine 150 + 150	48 and 15	24 8 ± 11 1	27
10	150 + 150	48 and 15	29 0 ± 2 8	
11	Fibrinolysin[3] [4]	½	46 38	38
12	Fibrinolysin[3] [4]	½	43 24	

1) Control rats were injected with saline
2) 3 rats in test and control groups
3) Dose 50 000 MSD U/kg (40 Casein U/kg)
4) 2 rats in test group

induced rat paw oedema when injected intraperitoneally 30 minutes previously Experiment 3 in addition demonstrated a potentiation of the effect of noradrenaline by the MAO inhibitor, iproniazid Iproniazid alone had no significant effect The effects of the adrenergic substances were of rather short duration When noradrenaline was injected 3 hours before

Table 3

Effects of kininogen fractions, total kininogen and prekallikrein in rat plasma of various substances injected intraperitoneally

Intraperitoneal injections 1.0 ml/100 g.

I The kininogen fraction activated by an excess amount of rat plasma kallikrein preparation

II The kininogen fraction activated by an excess amount of rat urine kallikrein preparation

IIIa Total kininogen activated by rat plasma kallikrein followed by rat urine kallikrein

IIIb Total kininogen activated by acetone (20 % v/v) and then evaporation of the acetone

The batches of kallikrein preparations were tested for their kinin releasing activity in comparison with a standard batch

Female rats were used in experiment 4 in the other experiments male rats

Blood was collected 30 minutes after the intraperitoneal injections except for reserpine when a time interval of 24 hours was used

For further details see text and BRISEID *et al* (1970)

Experiment number	Kininogen batch number	Number of rats	Intraperitoneal injection mg/kg	Kinin released per ml plasma as µg bradykinin				Prekallikrein test/standard
				I	II	IIIa	IIIb	
1	1	5	Adrenaline 0 75	1 6	1 4	2 3	1 8	1 1
	2	13	Saline	1 7	1 4	2.8	2 0	
2	3	10	Noradrenaline 0 50	1 3	1 3	2 2	2 2	1 1
	4	9	Saline	1 3	1 2	2.3	2 2	
3	5	7	Reserpine 5 0	1 6	1 4	2 1	2 1	1 1
	4	9	Saline	1 4	1 4	2 0	2 1	
4	6	5	Fibrinolysin[1]	2 0	1 9	3.3		
	7	6	Saline	1 8	2 0	3 0		

1) Dose 50 000 MSD U/kg (40 casein U/kg)

carrageenin, only a weak inhibition was observed (experiment 4) Depletion by drugs of stores of monoamines reduced the degree of oedema development In table 2 the results of inhibition experiments are presented with different doses of reserpine and of guanethidine An amount of reserpine of 1 mg/kg was required to obtain a significant reduction of the paw oedema In table 2 the results of inhibition experiments with the plasmin preparation fibrinolysin® are also included

Experiment 5 in table 1 shows that iproniazid counteracted the oedema inhibition caused by reserpine, which is in agreement with the observation of previous investigators that an inhibition of MAO protects the stores of

Table 4

Determination of the serotonin blood level in rats
Influence of noradrenaline and amine depleting drugs injected intraperitoneally, and of paw injections of carrageenin

Paw injections in rats in experiments 1 2 4 and 5 0 1 ml per rat of a 1 % (w/v) suspension of carrageenin in saline
In experiments 4 and 5 to correlate oedema inhibition values and serotonin levels, blood was collected as soon as possible after the oedema experiment
In experiment 3 plasma was separated from two of the three blood specimens in each group and the serotonin concentration was determined in the plasma phase as well
For further details see methods

Experiment number	Number of rats	Pretreatment	Time in hours before blood collection	Serotonin µg/ml		Rat paw oedema inhibition per cent Mean ± S D
				Blood Mean ± S D	Plasma[1]	
1	5	Carrageenin	4½	1 64 ± 0 49		
	5	None	–	1 76 ± 0 29		
2	5	Carrageenin	4½	1.52 ± 0 24		
	6	None	–	1 75 ± 0 07		
3	3	Noradrenaline 0 5mg/kg	½	1 96 ± 0 05	0 170, 0 123	
	3	Saline	½	1 74 ± 0 07	0 099, 0 137	
4	4	Reserpine 1.5mg/kg	24	0 056 ± 0 009		38 5 ± 21 4
	3	Reserpine 0 5mg/kg	24	0 068 ± 0 007		16 0 ± 6 6
	4	Reserpine 0 15mg/kg	24	0 52 ± 0 26		-14 8 ± 23 0
5	5	Guanethidine 2×15Omg/kg	53 and 20	1 72 ± 0 48		24 8 ± 11 1
	5	Saline	53 and 20	1 53 ± 0.33		

[1] Two rats only in test and control group in experiment 3

monoamines against depletion (CARLSSON *et al* 1957) The same experiment further shows that the inhibitory effects of reserpine and tyramine are additive, possibly indicating a direct effect of tyramine in its inhibition of the paw oedema

Table 3 shows that adrenaline, noradrenaline, reserpine and plasmin in anti inflammatory concentrations do not significantly influence the levels of kininogen fractions, total kininogen or prekallikrein in rat plasma The high kininogen values obtained in experiment 4 probably reflect the use of female Wistar rats, which, in our experience, have a somewhat higher level of plasma kininogen than male Wistar rats

Table 4 shows the results of determinations of the blood concentration of serotonin, which is normally practically identical with the amount present in the platelets Experiments 1 and 2 indicate a slight reduction in the serotonin level caused by the pedal injection of carrageenin This tendency, which has also been observed in other experiments, might indicate a local consumption of serotonin platelet stores

The intraperitoneal injection of noradrenaline does not significantly influence the blood concentration of serotonin (experiment 3) Simultaneous determinations of the plasma concentration of serotonin do not indicate a release of platelet serotonin caused by noradrenaline

Experiment 4 of table 4 clearly demonstrates the lack of correlation between the anti-oedema effect of reserpine and the depletion of platelet serotonin Reference should also be made to the anti-oedema concentrations of reserpine shown in table 2, experiments 1 to 8

Experiment 5 of table 4 provides evidence that guanethidine has no effect on the level of blood serotonin under conditions which cause a significant reduction of oedema development

The results presented in table 5 demonstrate that no reduction in urokinase-induced proteolytic activity in rat plasma is observed after the injection of noradrenaline in an effective anti oedema concentration

Discussion

Carrageenin-induced rat paw oedema has been used as an inflammatory model by a number of investigators DI ROSA & WILLOUGHBY (1971) suggested that the inflammatory response consists of three distinct phases a first phase that can be attributed to the release of histamine and serotonin Kinins are probably responsible for the second phase The third phase which is assumed to be responsible for the maintenance of the enhanced vascular permeability, is assumed to be mediated by prostaglandins Observations made by WILLIS (1969) also support such a division of the carrageenin inflammation into relatively distinct phases

In the present work experiments carried out with drugs capable of pleting stores of monoamines in the rat (reserpine guanethidine tyram provided evidence that catecholamines or serotonin might be impor factors in the development of carrageenin induced rat paw oedema. Re: of experiments with sympatholytics and with reserpine led ROCHA E S (1964) to the assumption that catecholamines might participate in the ger of thermal oedema in the rat paw and BONTA & DE VOS (1967) stated normal catecholamine stores rather than normal histamine or serotonin st seem to be necessary for the development of kaolin induced rat paw oede

While reserpine is known to cause a long lasting depletion of stores noradrenaline adrenaline and serotonin the effect of guanethidine is so what more selective in favour of depletion of the catecholamines Guane dine can also lower serotonin stores but this effect is rather short las (CASS & SPRIGGS 1961) Experiment 5 in table 4 shows that guanethi left the platelet serotonin intact under experimental conditions which ca a significant reduction in the carrageenin induced oedema The results experiment 4 in the same table demonstrate that an almost total deple of platelet serotonin is obtained by 0 5 mg/kg of reserpine while 0 15 mg causes a partial depletion The fact that such amounts of reserpine en insignificantly or not at all inhibited the development of paw oedema (cf table 2) is strong evidence that in our experiments blood serotonin is an essential factor for the induction of carrageenin-oedema Tissue seroto however, was not examined and the possibility cannot be excluded serotonin from sources outside the blood and more resistant to reserp than platelet serotonin might participate in the development of rat p oedema It should be mentioned that the doses of reserpine and guanethid required to obtain oedema inhibition are of the same order of size as doses found by KUNTZMAN *et al* (1962) to be required for lowering content of catecholamines in the adrenal medulla which was the most re tant tissue This correlation might further support the theory that a minim amount of catecholamine must be available for an adequate developm of the inflammatory reaction The experiments certainly do not exclu the possibility of some unknown monoamine instead of catecholamines be involved in the inflammatory reaction Such a hypothetical substance pro ably has to be metabolized by MAO as pretreatment with iproniazid sign cantly reduces the reserpine induced inhibition of the paw oedema (table experiment 5)

The possibility that catecholamines are essential for the development carrageenin induced rat paw oedema together with the fact that pretreatm with catecholamines inhibits the oedema development might suggest th the same pathway of biochemical events is involved in the inflammato process and in the preventive mechanism Furthermore the oedema inhibiti

Table 5

Determination of plasminogen in rat plasma Effect of noradrenaline

Rats were injected intraperitoneally with noradrenaline 0 5 mg/kg 30 minutes before blood collection
Control rats were injected with the same volume of saline (1 0 ml/100 g)
Acid treated plasma specimens were incubated with casein and urokinase for 30 minutes Undigested casein was then precipitated with perchloric acid and the optical density in the supernatant was read A) at 275 mμ B) after adjusting the pH to 12 at 428 mμ Another sample from the same plasma specimen was incubated in parallel without urokinase The difference in optical density (Δ O D) between the two samples was taken as a measure of the plasminogen concentration in the plasma The mean value of Δ O D in the control group was set at 100 % activity, and all the other values were expressed as per cent of this
For further details see methods

		A		B	
Treatment	n	ΔO D.x10³ ± S E M	Plasminogen relative activity Per cent ± S E M	ΔO D x10³ ± S E M	Plasminogen relative activity Per cent ± S E M
Saline	13	355 ± 13	100 ± 4	101 ± 2	100 ± 2
Nor adrenaline	11	375 ± 14	106 ± 4	105 ± 3	104 ± 3

might reflect an acute activation and transitory depletion of an unknown factor participating in the chain of events leading to the development of oedema

It has previously been shown that serotonin in concentrations which inhibit carrageenin induced rat paw oedema is also able to cause a reduction in the levels of kininogen and prekallikrein in rat plasma (BRISEID *et al* 1971) The anti inflammatory effects of adrenaline and noradrenaline in the present experiments, however, can hardly be explained by an influence on the above mentioned parameters of the kinin system (table 3, experiments 1 and 2)

As mentioned in the introduction several investigators have described the activation of the fibrinolytic system by adrenaline or noradrenaline Thus a reduction in the plasminogen concentration might be expected during periods of increased fibrinolytic activity In our experiments however, we did not observe a fall in plasminogen in rat plasma after the injection of noradrenaline in an oedema inhibiting concentration (table 5) This does not necessarily exclude the possibility that the fibrinolytic system is involved The results in the papers referred to are based on determinations of the increase in total fibrinolytic activity in connection with a higher level of adrenergic activity, and plasminogen determinations were not included

Moreover, the enhancement of fibrinolysis was of rather short duration activity returning to preinjection level within 30 minutes (HOLEMANS 19
An activation by plasmin of kininogen via kallikrein is now well establis (VOGT 1964, WEBSTER & INNERFIELD 1965, HAUSTEIN & MARQUARDT 19 HENRIQUES *et al* 1966) Most investigations, however, were based on *in v* experiments and plasmin induced release of kinin *in vivo* is question (WEBSTER & INNERFIELD 1965, WENTZ *et al* 1971) In our experim plasmin does not alter the kininogen level (table 3, experiment 4) un conditions causing inhibition of rat paw oedema (table 2, experiments and 12) Some recent reports have provided evidence of another mechan through which plasmin might activate the kinin system According to KAP & AUSTEN (1971) active Hageman factor might partly undergo excha into fragments possessing a strong ability to activate prekallikrein but w a decreased ability to initiate the coagulation process Plasmin was fo to support the dissociation of active Hageman factor into such fragments

It should be pointed out that a plasmin induced activation of the ki system is not the only factor which might cause a plasmin induced incre in permeability The activation of the complement system through plas and the production of a permeability increasing polypeptide from one the early reacting components has been described in investigations on reditary angioneurotic oedema (DONALDSON 1968, KLEMPERER *et al* 19 DONALDSON *et al* 1969) It should be mentioned that carrageenin has a been reported to activate complement (WILLOUGHBY *et al* 1969)

REFERENCES

Bhalla T N J N Sinha K K Tangri & K P Bhargava Role of catecholamines inflammation *Eur J Pharmacol* 1970 13 90–96

Biggs R R G Macfarlane & J Pilling Observations on fibrinolysis Experimen activity produced by exercise or adrenaline *Lancet* 1947 1 402–405

Bonta I L & C J de Vos Significance of the kinin system in rat paw oedemas a drug effects on it *Eur J Pharmacol* 1967 1 222 225

Briseid K F C Arntzen & O K Dyrud Inhibition of carrageenin induced rat p oedema by substances causing a reduction of kininogen and prekallikrein in plasn *Acta pharmacol et toxicol* 1971 29 265–274

Briseid K O K Dyrud & S Öie Determination of factors of the kinin system in plasma *Acta pharmacol et toxicol* 1970 28 124–137

Brown J H H K Mackey D A Riggilo & N L Schwartz Studies on the acu inflammatory response II Influence of antihistaminics and catecholamines formaldehyde induced edema *J Pharmacol exp Therap* 1968 160 243–248

Carlsson A E Rosengren A Bertler & J Nilsson Effect of reserpine on the meta olism of catecholamines In *Psychotropic drugs* Eds S Garattini and V Ghet Elsevier Publishing Co Amsterdam 1957 363–372

Cass, R. & T L B Spriggs Tissue amine levels and sympathetic blockade after guanethidine and bretylium *Brit J Pharmacol* 1961, **17**, 442–450

Cho, M H & W Choy Induction of increased fibrinolytic activator in plasma by various drugs *Thromb Diath haemorrh* 1964, **11**, 372–392

Crunkhorn P. & S C R Meacock Mediators of the inflammation induced in the rat paw by carrageenin *Brit J Pharmacol* 1971, **42**, 392–402

Di Rosa, M & D A Willoughby *Mediators of increased vascular permeability* and cellular emigration in carrageenin foot oedema *Naunyn Schmiedebergs Arch Pharmacol* 1971, **269**, 482.

Donaldson, V H C'1 activation in hereditary angioneurotic edema plasma role of urokinase and inhibitors *J appl Physiol* 1968, **25**, 416–424

Donaldson V H, O D Ratnoff, W D Da Silva & F S Rosen Permeability increasing activity in hereditary angioneurotic edema plasma II Mechanism of formation and partial characterization *J clin Invest* 1969, **48** 642–653

Eisen V & W Vogt Plasma kininogenases and their activators In *Bradykinin, kallidin and kallikrein* Handbook of experimental pharmacology, volume XXV Ed E G Erdos Springer Verlag, Berlin, Heidelberg New York 1970, 82–130

Fekete M & A M Kürti Effect of monoamine oxidase inhibitors on rat paw edema induced by various phlogistic agents *Eur J Pharmacol* 1970, **10**, 268–276

Genton, E, F Kern Jr & K. von Kaulla Fibrinolysis induced by pressor amines *Amer J Med* 1961, **31**, 564–571

Gozsy, B & L. Kátó Delayed inflammatory response due to catecholamines and serotonin *Nature* 1966, **212**, 1049–1050

Haustein, K. O & F Marquardt Über den Einfluss von Fibrinolytica auf die Aktivierung des Kininsystems durch Fibrinolysevorgange *Acta biol med ger* 1966, **16**, 658–664

Henriques, O B, A A C Lavras, M Fichman & Z P Picarelli Plasma enzymes that release kinins *Biochem Pharmacol* 1966, **15** 31–40

Holemans, R. Enhancement of fibrinolysis in the dog by injection of vasoactive drugs *Amer J Physiol* 1965, **208**, 511–520

Jacobsen, C. D The caseinolytic method for determination of plasminogen plasmin in human plasma and serum *Scand J clin Lab Invest* 1964, **16** 372–382

Kaplan, A P & K F Austen A prealbumin activator of kallikrein II Derivation of activators of prekallikrein from active Hageman factor by digestion with plasmin *J exp Med* 1971, **133**, 696–712

Klemperer, M R, V H Donaldson & F S Rosen Effect of C1 esterase on vascular permeability in man Studies in normal and complement-deficient individuals and in patients with hereditary angioneurotic edema *J clin Invest* 1968, **47**, 604–611

Kuntzman R, E Costa, G L Gessa & B B Brodie Reserpine and guanethidine action on peripheral stores of catecholamines *Life Sci* 1962, no 3, 65–74

Pohala, M J, C Y Yen & H O Singher Effect of neuroactive drugs on production of fibrinolytic activity *Amer J Physiol* 1962, **202** 984–986

Rocha E Silva, M Chemical mediators of the acute inflammatory reaction *Ann N Y Acad Sci* 1964 **116** 899–911

Spector, W G & D A Willoughby Vaso-active amines in acute inflammation *Ann N Y Acad Sci* 1964, **116** 839–846

Vogt, W Kinin formation by plasmin, an indirect process mediated by activation of kallikrein *J Physiol* 1964 **170**, 153–166

Webster, M E & I Innerfield Interrelationship of human plasma kallikrein and plasmin in inflammation *Enzymol biol clin* 1965 5 no 3 129 148

Wentz, U, G Seidel, C Meyer Burgdorf & E Habel Formation of kinins during hyper fibrinolysis *in vivo? Naunyn-Schmiedebergs Arch Pharmacol* 1971, 270 (suppl)

Willis, A L. Release of histamine, kinin and prostaglandins during carrageenin induced inflammation in the rat In *Prostaglandins, peptides and amines* Eds P Mantegazza and E W Horton Academic Press, London and New York 1969, 31-38

Willoughby, D A, E Coote & J L Turk Complement in acute inflammation *J Pathol* 1969, 97, 295-305

Yuwiler, A, S Plotkin, E Geller & E R Ritvo A rapid accurate procedure for the determination of serotonin in whole human blood *Biochem Med* 1970 3 426-431

Acta pharmacol et toxicol 1973, 32, 193–204.

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Substrates for Kinin-Releasing Enzymes in Human Plasma

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Abstract Indirect evidence has been provided of the presence of 3 kininogen fractions. The average amounts of kinin released by human plasma kallikrein (1.2 µg/ml plasma) and by human urine kininogenase (2.4 µg/ml plasma) when added up exceeded the amount of kinin released by combined use of the two enzyme preparations (3.0 µg/ml plasma). Methods were as described by BRISEID *et al.* (1968). It is suggested that plasma kallikrein released kinin from 2 kininogen fractions S1' and S1'', and that urine kallikrein released kinin from 2 kininogen fractions S1'' and S2. Repeated incubation with each of the kininogenase preparations did not increase the yield of kinin. Soybean trypsin inhibitor did not reduce the amount of kinin released by urine kallikrein. In control experiments a leucine aminopeptidase preparation transformed kallidin to bradykinin but did not increase the kinin activity of the urine kallikrein incubates. Experiments carried out with plasma kallikrein and padutin in 60° heated plasma supported the assumption of the 3 kininogen fractions.

Key words: Kinins – kininogen – kallikrein

In a previous paper indirect evidence was provided of the presence o
3 kininogen fractions or, more precisely, 3 functional states of kininogen i
rat plasma (BRISEID *et al.* 1970). The theory was advanced that acetone
activated rat plasma kallikrein released kinin from a kininogen fraction nc
activated by rat urine kallikrein (substrate 1') as well as from another fractio
which was also activated by urine kallikrein (substrate 1''). The urine ka
likrein preparation, on the other hand, released kinin from a kininoge
fraction not activated by the plasma kallikrein preparation (substrate 2) an
from the fraction also activated by plasma kallikrein (substrate 1''). Th
experiments were carried out with non heated, citrated rat plasma, accord
ingly with kininogenase inhibitors and prekininogenases intact.

The experiments presented in this paper were designed to find out wheth
similar evidence could also be obtained for the presence in human plasm
of 3 functional states of kininogen, referred to throughout as kininoge
fractions.

Technique

A Materials and assays

Human plasma kininogen and *human plasma kallikrein* were prepared as described by BRISEID *et al* (1968) The only difference was that the acetone was evaporated *in vacuo* from the kallikrein preparation This was done before EDTA 2Na was added for the elimination of the kininase activity

Human urine kininogenase Freshly voided morning urine from 3 healthy men was pooled and dialyzed against running tap water for 24 hours Acetone was then added (12 volumes to 10 volumes of urine) and after 10 minutes the acetone was removed by evaporation *in vacuo* The remaining fluid was filtered and passed through a Sephadex G 100 column The filtrate was concentrated *in vacuo* to about 1/10 of the volume of the dialyzed urine EDTA 2Na (4 mg/ml) was added and the pH adjusted to 7 3 After incubation for 30 minutes at 37° the urine preparation was stored at −20°

Hog pancreas kallikrein Padutin R® in ampoules of 10 biol units Bayer A G Leverkusen Germany The preparation was found to be free from kininase

Reagents Bradykinin Sandoz, A G , Basel Switzerland *Ethylenediamine tetra acet c acid disodium salt (EDTA 2Na)* Titriplex III, Merck A G , Darmstadt Germany *Leu cine aminopeptidase* and *Soybean trypsin inhibitor* Worthington Biochemical Corp Freehold New Jersey U S A *α N Benzoyl L-arginine ethyl ester (BAEE)* and *p tosyl L arginine methyl ester (TAME)* Sigma Chemical Company, St Louis MO U S A

Assays The kinin determinations were carried out on the isolated rat uterus as bracketing assays with a dose ratio of 3 2 Bradykinin was used as standard substance

B Methods

Determination of the kininogen fraction activated by incubation with plasma kall krein To 1 00 ml kininogen preparation 0 50 ml of plasma kallikrein preparation was added and the mixture incubated at 37° for 10 minutes From the incubate 0 20 ml was withdrawn and transferred to 2 5 ml boiling saline and the mixture heated for 5 minutes in a boiling waterbath The mixture was then diluted with saline to 5 00 ml and the solution was assayed at once or kept at 4° for not more than 24 hours or stored at −20° for some days before assay

Determination of the fractions activated by incubation with urine kininogenase To 1 00 ml kininogen preparation 1 00 ml of the urine preparation was added and the mix ture was incubated at 37° for 15 minutes The further procedures were as described above for the determination of the kininogen activated by plasma kallikrein

Determination of the fraction activated by incubation with hog pancreas kallikrein (padutin) To 1 00 ml kininogen preparation was added 3 2 units padutin in 0 08 ml d s tilled water and the mixture incubated for 30 minutes at 37° The further procedures were as described above for the determination of the kininogen activated by plasma kallikrein

Determination of total kininogen was carried out by acetone activation as described previously (BRISEID *et al* 1967)

Comments on the Technique

Determination of the kininogen fraction activated by incubation with plasma kalli krein The concentration-effect and time-effect data have been published previously (BRISEID *et al* 1968) It was then demonstrated that less than 0 2 ml kallikrein prepara

tion per ml substrate sufficed for maximum kinin release in non heated plasma The concentration-effect values shown in table 1 extend the results of the above mentioned paper No significant increase in kinin release took place over the concentration range 0.2 ml to 6 ml of enzyme preparation per ml substrate Table 2 gives the results of a typical time-effect experiment Under the conditions chosen 4 minutes were found sufficient for maximum kinin release

Determination of the fraction activated by incubation with urine kininogenase The results of 2 typical concentration-effect experiments are shown in table 1 It can be seen that no further kinin release was obtained by increasing the amount of enzyme preparation beyond 1 ml per ml plasma. The time-effect data in table 2 show that about 15 minutes sufficed for maximum kinin release It should be mentioned that the urine preparation showed weak esterolytic activity when tested against benzoyl L-arginine ethyl ester (BAEE) or p tosyl L-arginine methyl ester (TAME) the esterase/kininogenase ratio being about 1/40 of that of human plasma kallikrein

Determination of the fraction activated by incubation with hog pancreas kallikrein (padutin) The concentration effect and time effect data have been published previously (BIELTVEDT & BRISEID 1967) It was then found that about 2 units of padutin per ml

Table 1

Concentration effect experiments with different kininogenase preparations

Plasma kininogen preparations Citrated plasma with EDTA 2Na, 4 mg/ml plasma
Two batches tested for each kininogenase preparation

Incubation periods Plasma kallikrein, 10 minutes
Urine kininogenase, 15 minutes
Padutin, 30 minutes

Total kininogen determined by acetone activation (BRISEID *et al* 1967)
For further details see text

(1) Kinin released per ml plasma as μg bradykinin
(2) Kinin released per ml plasma as per cent of total kininogen
(3) Releasing enzyme in ml/ml plasma (plasma kallikrein and urine kininogenase) or in units/ml plasma (padutin)

Plasma kallikrein			Urine kininogenase			Padutin		
(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
1 3	30	0 25	2 3	52	0 2	1 9	40	1 0
1 2	28	0 5	2 6	59	0 4	2 5	53	2 0
1 4	33	1 0	3 1	70	0 7	2 5	53	4 0
1 5	37	2 0	3 1	70	1 0	2 5	53	5 0
			3 3	75	2 0	2 5	53	6 0
1 4	27	0 2	2 7	63	0 5	1 8	38	1 0
1 3	25	0.5	2 9	67	1 0	2 3	49	2 0
1 2	23	1 0	2 9	67	2 0	2.5	53	3 0
1 5	29	2 0	2 8	65	4 0	2 5	53	4 0
1 3	25	6 0				2 8	60	5 0

Table 2

Time effect experiments with different kininogenase preparations

Plasma kininogen preparation Citrated plasma with EDTA 2Na, 4 mg/ml plasma
Enzyme concentrations Plasma kallikrein, 1 ml per ml plasma
Urine kininogenase, 1 ml per ml plasma
Padutin, 3 2 units per ml plasma
Total kininogen determined by acetone activation (BRISEID *et al* 1967)
For further details see text

(1) Kinin released per ml plasma as µg bradykinin
(2) Kinin released per ml plasma as per cent of total kininogen
(3) Incubation period in minutes

Plasma kallikrein			Urine kininogenase			Padutin		
(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
1 1	26	2	1.5	38	2	2.5	51	7.5
1 2	29	4	2 0	51	4	2 8	57	15
1 2	29	7.5	2 2	56	7 5	2 8	57	30
1 2	29	15	2.5	66	15	2 8	57	60
1 2	29	30	2 6	67	30	2.5	51	120
1.2	29	60	2 6	67	60	2.5	51	180

substrate were required for maximum kinin release and similar results were obtained in the present work (table 1) From the time-effect data in the previous paper (BIELTVEDT & BRISEID 1967) and in the present work (table 2) an incubation time of 30 minutes was chosen

Results

Two preparations of human plasma kininogen were tested in most of the experiments shown in table 3 For each of the batches the data given in the table represent typical results from several experiments Human plasma kallikrein was tested together with human urinary kininogenase and also together with hog pancreas kallikrein (padutin) In most of the experiments the total kininogen was determined in parallel by the acetone activation method (BRISEID *et al* 1967) The percentages shown in the table for the extent of release observed with plasma kallikrein and the glandular kininogenases demonstrate that full kinin release was never obtained by the combined use of the enzyme preparations Only about 80 per cent of the total kininogen was released This result differed from the results previously obtained in experiments with rat plasma (BRISEID *et al* 1970), in which maximum release was achieved by the combined use of rat plasma kallikrein and rat urine kallikrein

Experiments with plasma kallikrein and urine kininogenase in non heated plasma The results of experiments I, II, III and IV in table 3 show the kinin release obtained by repeated incubations of citrated EDTA-plasma with human plasma kallikrein or with urine kininogenase, as well as the release obtained by the combined use of the 2 kininogenase preparations

Table 3

Kinin release in human citrated EDTA plasma by excess amounts of different kininogenase preparations

Kininogenase preparations

HP kall Acetone activated human plasma kallikrein
HU kall Human urine kininogenase
Padutin Hog pancreas kallikrein
The kininogenase preparations were used in amounts of 0.5 ml, 1 0 ml and 3.2 units respectively per ml kininogen preparation

LAP Leucine aminopeptidase was used in an amount which rapidly converted 2 μg kallidin in 2 ml 0 1 M tris buffer containing 4 mg EDTA 2Na per ml to bradykinin

SBTI Soy bean trypsin inhibitor, 0,5 mg per ml kininogen preparation was used

Saline 0 9 % sodium chloride solution was added to a dilution of the kininogen preparations to 1+4 (v/v)

All incubations were carried out at 37° Total kininogen was determined by acetone activation (BRISEID *et al* 1967)
For further details see text

Experiment	Batch of plasma kininogen preparation	(1) Kinin released per ml plasma as μg bradykinin (2) Kinin released per ml plasma as per cent of total kininogen (3) Incubation period in minutes							
		Incubation with	*(1)*	*(2)*	*(3)*	*Incubation with*	*(1)*	*(2)*	*(3)*
I	A	1 HP kall	1 2	31	10	1 HP kall	1 2	25	10
		2. HU kall	3 1	79	15	2 Padutin	3 7	79	30
	B	1 HP kall	1 1	26	10	1 HP kall	1 1	26	10
		2 HU kall	3 1	74	15	2 Padutin	3 7	88	30
II	A	1 HU kall	2 3	59	15	1 Padutin	3 1	66	30
		2 HP kall	2 9	74	10	2 HP kall	3 8	81	10
	B	1 HU kall	2 7	64	15	1 Padutin	3 2	76	30
		2 HP kall	3 0	71	10	2 HP kall	3 4	81	10
III	A	1 HP kall	1 2	31	10	1 HP kall	1 2	26	10
		2 HP kall	1 2	31	10	2 HP kall	1 3	28	10
	B	1 HP kall	1 4	33	10	1 HP kall	1 1	26	10
		2 HP kall	1 2	29	10	2 HP kall	1 4	31	10

Table 3
(continued)

Experiment	Batch of plasma kininogen preparation	Incubation with	(1)	(2)	(3)	Incubation with	(1)	(2)
		(1) Kinin released per ml plasma as µg bradykinin; (2) Kinin released per ml plasma as per cent of total kini; (3) Incubation period in minutes						
IV	A	1 HU kall	2.2	69	15	1 Padutin	3.1	66
		2 HU kall	2.2	69	15	2 Padutin	3.7	79
	B	1 HU kall	2.7	64	15	1 Padutin	3.2	76
		2 HU kall	2.9	69	15	2 Padutin	3.1	74
V	A	1 HU kall	2.1	66	15	1 Padutin	3.0	61
		2 LAP	2.2	69	30	2 LAP	3.2	65
VI	A	1 HP kall	1.3	27	10	–	–	–
		2 LAP	1.3	27	30			
VII	A	HP kall	1.4	36	10	HP kall	1.3	27
		SBTI+HP kall	*0.1*	*<3*	*10*	*SBTI+HP kall*	*<0.1*	*<2*
	B	HP kall	1.1	–	10	HP kall	1.2	26
		SBTI+HP kall	0.3	–	10	SBTI+HP kall	0.2	<4
VIII	A	HU kall	2.6	60	15	Padutin	3.0	61
		SBTI+HU kall	2.4	55	15	SBTI+padutin	3.2	65
	B	*HU kall*	*2.7*	*64*	*15*	Padutin	*3.0*	*63*
		SBTI+HU kall	2.8	67	15	1 SBTI+padutin	2.3	48
						2 SBTI+padutin	2.9	60
IX	A	Saline	0.1	3	60			
		SBTI+saline	<0.1	<3	60			
	B	Saline	0.1	2	60			
		SBTI+saline	0.1	2	60			

Experiments V and VI were carried out to ascertain whether ami peptidases of plasma under the experimental conditions used were capa of transforming released kallidin to bradykinin, which is about twice as ac in the rat uterus assay. It has previously been found that some leucine ami peptidase (LAP) preparations cause a transformation (BRISEID *et al.* 19 and the enzyme used in the present work was controlled for such an effect

It is generally accepted that soy bean trypsin inhibitor (SBTI) inhi plasma kallikrein, but not glandular kallikrein. The results of experime

VII and VIII, in agreement with this, show that SBTI inhibited the acetone-activated plasma kallikrein preparation used but not the urine preparation

Experiment IX of table 3 shows that an insignificant kinin release took place on dilution of the plasma kininogen preparations with saline (1+4 v/v) and subsequent incubation for 60 minutes

From the experiments shown in table 3 and similar experiments it could be calculated that plasma kallikrein on an average released 1 2 µg kinin as bradykinin per ml plasma substrate, urine kininogenase released 2 4 µg and the two enzyme preparations together released 3 0 µg of kinin

Experiments with plasma kallikrein and padutin in non-heated plasma The results of the plasma kallikrein padutin series of experiments shown in table 3 differed from the plasma kallikrein urine kininogenase series in one important respect while the amount of kinin released by the urine preparation could always be increased to some extent by further incubation with plasma kallikrein, the hog pancreas kallikrein preparation, padutin, usually accounted for almost the same release of kinin as was obtained by the combined use of plasma kallikrein and padutin In some experiments a repeated incubation with padutin was necessary to obtain maximum release (table 3, experiment IV, batch A), in other experiments one single incubation period sufficed (table 3, experiments IV, batch B)

Experiments with plasma kallikrein and padutin in 60°-heated plasma When citrated EDTA-plasma heated for one hour at 60° to inactivate pre-kallikrein and kallikrein inhibitors was repeatedly incubated with plasma kallikrein or padutin the amounts of kinin released increased stepwise (table 4 experiment I, a, b) The same experiment in table 4 also gives the results of the kinin release obtained with combinations of the two kininogenase preparations It can be seen that the amount of kinin released was larger when plasma kallikrein was used before padutin than for the reversed sequence (table 4, experiment I, c d) The small difference was regularly observed in a number of similar experiments

Experiments with plasma kallikrein and urine kininogenase in 60°-heated plasma When citrated EDTA plasma heated for one hour at 60° was incubated with an excess amount of the urine kininogenase preparation a maximum kinin release was obtained and further incubation with this enzyme or with padutin did not increase the yield of kinin significantly (table 4, experiment II, a b) When plasma kallikrein was used after the urine preparation some increase in the yield of kinin was noted (table 4, experiment II c) but the yield was clearly larger when the incubation with plasma kallikrein was carried out first (table 4, experiment IId) This was in accordance with the results claimed for plasma kallikrein and padutin (table 4, experiment I, a, b c, d) and this was also seen for padutin plasma kallikrein combinations in experiment II, e, f of table 4 While padutin did not increase

Table 4.

Kinin release obtained by excess amounts of different kininogenase preparations in human citrated EDTA plasma heated at 60° for one hour

The kininogen preparations used repeatedly incubated with fresh amounts of the same enzyme preparation or incubated with two enzymes the sequence given by the numbers … batches of plasma kininogen preparation, … lasma calculated as bradykinin … the level of the values in experiment I the amount of kinin released by plasma kallikrein being used as ' interior standard For further details see previous tables and text

Experiment	Incubation with	µg bradykinin per ml plasma	Experiment	Incubation with	µg bradykinin per ml plasma
Ia	1 HP kall	1.3	IIa	1 HU kall	20
				2 HU kall	21
	2 HP kall	15	IIb	1 HU kall	20
	3 HP kall	17		2 Padutin	20
Ib	1 Padutin	15	IIc	1 HU kall	20
				2 HP kall	2.3
	2 Padutin	17	IId	1 HP kall	13
	3 Padutin	19		2 HU kall	26
Ic	1 HP kall	13	IIe	1 HP kall	1.3
				2 Padutin	26
	2 Padutin	22	IIf	1 Padutin	18
				2 HP kall	2.3
Id	1 Padutin	1.5	IIg	1 Padutin	1.8
	2 HP kall	19		2 HU kall	22

the yield of kinin released by the urine kininogenase (table 4, experiment IIb), incubation in a reversed sequence caused a significant increase in the amount of kinin (table 4, experiment IIg)

Discussion

An extensive literature covers the field of kininogen fractions, and the surveys of HABERMANN (1970) and PIERCE (1970) discuss the most important

communications Most of the reports are concerned with the isolation and purification of kininogen or kininogen fractions and are consequently of no direct relevance to the present work which had the purpose of providing indirect evidence for the existence of different functional states of kininogen The existence of (at least) 2 functional different kininogens in human plasma must be considered as well established (MARGOLIS & BISHOP 1963, EISEN 1966, JACOBSEN 1966, VOGT 1966, VOGT *et al* 1967, VOGT & WAWRETSCHEK 1968)

Experiments with human plasma kallikrein and human urine kininogenase in non heated plasma In experiments I and II of table 3 the kinin release obtained with plasma kallikrein and with urine kininogenase were determined, as well as the release values obtained by the combined use of the 2 kininogenase preparations The fact that additional incubations of the kininogen preparation with plasma kallikrein, or urine kallikrein, did not increase the yield of kinin (table 3, experiments III and IV) suggests that the cessation of kinin release observed in the first-mentioned experiments must be ascribed to the presence of at least 2 different kininogen fractions, substrates 1 and 2 The added amounts of kinin released in the kininogen preparation by plasma kallikrein and urine kininogenase exceeded the amount of kinin released by the combined use of the enzyme preparations (table 3, experiments I and II) This observation might suggest that the two crude kininogenase preparations partly activated different kininogen fractions but also partly the same fraction Thus 3 different kininogen fractions must have been present The observation of "extra" kinin release might, however, also reflect an overwhelming of kininogenase inhibitors present in the kininogen preparation by one or both of the kininogenase preparations used Thus 2 kininogen fractions only would suffice to explain the results The fact that repeated incubation of the substrate with each of the kininogenase preparations did not increase the yield of kinin (table 3, experiments III and IV) suggested that the *direct* effect of the kininogenase preparations cannot explain the extra kinin release It does not, however, exclude the possibility that this release might be due to an activation of prekininogenases in the substrate In this case the first incubation with kininogenase preparation would exert a stronger effect than the subsequent incubations

The possibility that the urine preparation acted through activation of SBTI sensitive plasma kininogenases was excluded in view of the experiments in which it was shown that SBTI did not reduce the amount of kinin released by the glandular kallikrein The plasma kallikrein preparation on the other hand, was almost completely inhibited (table 3 experiments VII and VIII)

Experiments with human plasma kallikrein and padutin in non heated plasma The fact that padutin, contrary to the urine preparation caused almost the same kinin release as was achieved by the combined use of plasma

kallikrein and padutin could be interpreted in different ways For example inhibitors present in the substrate might be more effective against the urine kininogenase preparation than against padutin Different reaction rates between kininogen fractions, inhibitors and enzymes would then determine the extent of kinin release The fact that padutin did not activate kininogen to the same extent in plasma heated at 60° for one hour (see below) seemed to contradict such an explanation If it were correct then one must assume that the heating procedure in addition to a destruction of inhibitors, to some extent also altered the kininogen fraction in question, leaving it less accessible to padutin

Another explanation seemed to be that padutin slowly caused an activation of a plasma prekininogenase The experiments carried out in the presence of SBTI, however, made such an explanation somewhat less likely In several experiments in addition to those shown in table 3 (experiments VII and VIII) it was found that SBTI did not inhibit the effect of padutin

Experiments with plasma kallikrein and padutin in 60°-heated plasma It is commonly accepted that heating at 60° for one hour destroys pre kallikrein in human plasma. Experiments carried out with such a substrate should accordingly provide information about a possible *indirect* effect as an explanation for the surplus in kinin release observed by the use of the enzyme preparations separately when compared to the release observed after their use in combination However, such a heating procedure will also, at least partly, destroy kallikrein inhibitors, and a *direct* unspecific effect of each of the kininogenases on the substrates after heating must be taken into consideration The increases in kinin release shown in table 4, experiment I, a b, by repeated incubations with plasma kallikrein or padutin probably reflect such unspecific effects

The larger yield of kinin which was found when plasma kallikrein was used before padutin (table 4, experiment I, c, d and experiment II e f) and which was also observed when padutin was replaced by urine kininogenase (experiment II, c, d) probably reflects the release of bradykinin by the plasma enzyme and kallidin by the glandular enzymes

When the results of the combination experiments Ic and Id and also of IIe and IIf in table 4 are corrected for unspecific release by plasma kallikrein and padutin and the kallidin released by padutin then is calculated according to bradykinin (activity ratio kallidin/bradykinin in the rat uterus assay set at 53 %, the average ratio observed in our experiments), it is found that the amount of kinin released by plasma kallikrein together with the amount released by padutin exceeds the amount released by combinations of the two enzymes, thus providing evidence for a third functional kininogen fraction The differences were not considerable (0 4–0 6 μg/ml plasma) but were regularly seen in different batches of citrated EDTA plasma

Experiments with plasma kallikrein and urine kininogenase in 60°-heated plasma In contrast to the results with padutin no significant unspecific release could be detected by repeated incubation with the urine kininogenase preparation or with padutin when used after the urine preparation (table 4, experiment II, a, b) On the contrary, an increase in the yield of kinin was observed when urine kininogenase was used after padutin (table 4, experiment II g) The results thus suggest that the same release is obtainable by the urine preparation alone, as could be achieved by the combined use of padutin and plasma kallikrein The increase in kinin release observed by incubation with plasma kallikrein after the urine preparation might reflect the presence of a small amount in the plasma kallikrein preparation of an amino peptidase capable of transforming kallidin to bradykinin (table 4, experiment IIc) If present, such an effect would also contribute to the results in experiments IIf and Id

When the results of the combination experiment II, c, d in table 4 were corrected for unspecific release by plasma kallikrein and the kallidin released by the urine kininogenase calculated to bradykinin as was done above for padutin, the data fitted reasonably well with the assumption that the urine preparation released as much kinin alone, as was obtained by the combined use of the two enzyme preparations

These results, together with the fact that the urine kininogenase preparation increased the yield of kinin when used after padutin while padutin did not increase the yield of kinin obtained by the urine preparation (experiment II, b, g), lend support for the assumption of the presence of 3 functional kininogen fractions in human citrated EDTA-plasma

Conclusion The experiments carried out with human citrated EDTA-plasma, non-heated and heated at 60°, in combination with acetone-activated human plasma kallikrein, with hog pancreas kallikrein (padutin) and with a kininogenase preparation from human urine can be interpreted as providing indirect evidence in favour of the presence of 3 functional kininogen fractions In addition some kininogen remained which could not be activated by the enzymes mentioned above under the conditions used, but which released kinin by acetone activation of non-heated plasma In accordance with the terminology used in connection with rat plasma kininogen (Briseid *et al* 1970) the fractions activated by plasma kallikrein might be designated as substrates 1′ and 1″, while the urine kininogenase preparation in addition activated a fraction designated as substrate 2 Only substrates 1″ and 2 were activated by padutin in 60° heated plasma

The indirect evidence presented in this study requires further work on purification experiments and biochemical data It should be taken into consideration, however that recent reports from work on bovine plasma have demonstrated the presence of two kininogens in the high molecular kininogen

fraction in addition to the kininogen fraction of lower molecular weight (YANO *et al* 1970 & 1971; SUZUKI *et al* 1971)

REFERENCES

Bieltvedt, G & K Briseid An assay method for padutin® (hog pancreas kallikrein) based on the *in vitro* release of kinin in human plasma *Acta pharmacol et toxicol* 1967, **25**, 190–196

Briseid, K, O K Dyrud & F C Arntzen Determination of prekallikrein in human plasma *Acta pharmacol et toxicol* 1968, **26**, 395–401

Briseid, K, O K. Dyrud & F C Arntzen Substrates for kininreleasing enzymes in rat plasma *Acta pharmacol et toxicol*, 1970, **28**, 138–144

Briseid, K, O K Dyrud & S F Rinvik The release of kinins in human plasma substrates *Acta pharmacol et toxicol* 1966, **24**, 39–49

Briseid, K, O K Dyrud & S F Rinvik *In vitro* estimation of the rate of release of kinin in human plasma *Acta pharmacol et toxicol* 1967, **25**, 201–203

Eisen, V Kininforming enzymes and substrates in human plasma *J physiol* 1966, **186** 133 P

Habermann, E Kininogens In *Handbook of experimental pharmacology* Bradykinin, kallidin and kallikrein Springer, Berlin, Heidelberg, New York 1970, **25**, 250–288

Jacobsen, S Substrates for plasma kinin forming enzymes in human, dog and rabbit plasmas *Brit J Pharmacol* 1966, **26**, 403–411

Margolis, J & E A Bishop Studies on plasma kinins I The composition of kininogen complex *Austral J exp Biol* 1963, **41**, 293–306

Pierce, J V Purification of mammalian kallikreins, kininogens, and kinins In *Handbook of experimental pharmacology* Bradykinin, kallidin and kallikrein Springer, Berlin, Heidelberg, New York 1970, **25**, 21–51

Suzuki, T, M Komiya, M Yano & H Kato Some properties of bovine high molecular weight kininogen In *International symposium on vasoactive peptides* Plenum Press, New York, in press

Vogt, W Demonstration of the presence of two separate kinin forming systems in human and other plasma In *Hypotensive peptides* Eds E G, Erdos, N Back and F Sicuteri Springer, Berlin, Heidelberg, New York 1966 185–192

Vogt W, G Garbe & G Schmidt Untersuchungen zur Existenz zweier verschiedener kininbildender Systeme in menschlichem Plasma *Arch exp Path Pharmakol* 1967, **256** 127–138

Vogt, W & W Wawretschek Weitere Untersuchungen zur Existenz zweier kininbildender Systeme in menschlichem Plasma *Arch exp Path Pharmacol* 1968, **260**, 223–230

Yano M, S Nagasawa & T Suzuki Evidence for the location of methionyl lysyl bradykinin moieties at the carboxyl terminus and inside of bovine kininogen I *Biochem Biophys Res Commun* 1970, **40**, 914–918

Yano M S Nagasawa & T Suzuki Partial purification and some properties of high molecular weight kininogen, bovine kininogen I *J Biochem* 1971, **69**, 471–481

Acta pharmacol et toxicol 1973, 32, 205–213

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Assay of Preparations of Acetone-activated Human Plasma Kallikrein by the Effects on Different Kininogen Fractions in Human Citrated Plasma

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Abstract Prekallikrein in human citrated plasma was activated by incubation with acetone, 17 or 25% (v/v) EDTA 2Na was added before or after the evaporation of acetone Determinations of the relative activities of the enzyme preparations were based on the release of kinin from the 3 functional states of kininogen in human citrated EDTA plasma as described by BRISEID *et al* (1973) Incubations were carried out at 37° or at 0° The kininogen 1/kininogen 2 activity ratios were the same at 37° and 0°, indicating that the kininogenase activities tested were the same The relative activities of the different kallikrein preparations varied with the type of kininogen used possibly reflecting the presence of more than one kininogenase Purification of the kallikrein combined with determinations of kininogen 1/kininogen 2 activity ratios must be carried out to solve the problem

Key words Kinins – kallikrein

Indirect evidence has been provided of the presence of 3 functional states of kininogen in human citrated EDTA plasma (BRISEID *et al* 1973) The kininogen fractions were given the designations 1′, 1″ and 2 in accordance with a previous study on similar fractions in rat plasma (BRISEID *et al* 1970) The present uncertainty about the presence of more than one kallikrein fraction in human plasma made it desirable to have at one's disposal methods for the determination of kallikrein activities, based not only on the release of kinin from the kininogen 1 fractions (BRISEID *et al* 1968, BRISEID *et al* 1973), but also from kininogen fraction 2 Estimations based on the last mentioned fraction have been complicated by the slow rate of kinin release, probably due to the low activities of the kallikrein preparations used In the present work a kininogen 2 preparation was provided by the elimination of fractions 1′ and 1″ through short-time incubation with a preparation of acetone-activated human plasma kallikrein The relative activities of

plasma kallikrein preparations activated by different acetone concentrations were determined both on the above mentioned kininogen 2 preparation and on the kininogen 1 fractions of untreated, citrated EDTA plasma

Technique

Human plasma kininogen (HP kin) was prepared by treating citrated plasma with EDTA 2Na as described by BRISEID *et al* (1968) 60° heated substrate *(HP kin/60°)* was prepared by heating for one hour in a waterbath The preparations were stored at −20°

Human plasma kininogen 2 (HP kin 2) 1 00 ml human citrated plasma was incubated for 20 minutes at 37° with human plasma kallikrein (HP kall/17 %) prepared without EDTA This was followed by incubation with 0 16 ml of a solution of EDTA 2Na 5 % (w/v) in distilled water for 30 minutes at 37° *HP kin 2/60°* was prepared by heating in a waterbath for one hour at 60° The preparations were stored at −20°

Human plasma kallikrein (HP kall/17%) was prepared as described by BRISEID *et al* (1968) by incubation of human citrated plasma for about 17 hours at room temperature with acetone 17% (v/v) The acetone was removed by evaporation *in vacuo* if not otherwise stated after 2 hours incubation at 37° with 0 08 ml of a solution of EDTA 2Na 5% (w/v) per ml citrated plasma

Human plasma kallikrein (HP kall/25%) was prepared as HP kall/17% with the following alterations 0 20 ml of a solution of lima bean trypsin inhibitor (LBTI) 1 % (w/v) in saline was added per ml of citrated plasma before the addition of acetone The evaporation of acetone took place *before* the incubation with EDTA 2Na, which lasted for 5 hours if not otherwise stated

Assay of HP kall based on the release of kinin from kininogen fractions 1

Kinin release temperature 0°

HP kin/60° and HP kall preparations were cooled to 0° in icewater To each of 3 one ml samples of HP kin/60° was added standard HP kall/25%, usually 0 05, 0 10 and 0 15 ml After incubation for 30 minutes at 0° a 0 20 ml sample was withdrawn from each incubate transferred to 2 5 ml boiling saline and heated for 5 minutes in a boiling waterbath The mixture was then diluted with saline to 5 00 ml and the kinin solution assayed at once or kept at −20° until assay The kinin values were calculated as µg bradykinin per ml plasma and a concentration effect curve was drawn Unknown HP kall preparations were assayed in parallel and their kininogenase activities calculated from the standard concentration effect curve

Kinin release temperature 37°

The procedure was as described above for kinin release at 0° with the following alterations the amounts of standard HP kall/25% added per ml HP kin/60° were usually 0 02 0 04 and 0 06 ml The kinin release period was 10 minutes

Assay of HP kall based on the release of kinin from kininogen fraction 2

Kinin release temperature 37°

To each of 3 one ml samples of HP kin 2/60° was added standard HP kall/25 %, usually 0 10 0 30 and 0 50 ml Each sample was then incubated for 30 minutes at 37° The further procedure was as described above for the assay of HP kall by the release of kinin from kininogen fractions 1

Kinin release temperature 0°

The procedure was as described above for kinin release at 37°, except for the period of kinin release, which was 4 hours

Assay of HP-kall based on the esterolytic activity against benzoyl arginine ethyl ester (BAEe) was carried out as described by LAAKE *et al* (unpublished results)

Reagents α *N Benzoyl L-arginine ethyl ester (BAEe)*, Sigma Chemical Company, St Louis, MO, U.S.A *Bradykinin*, Sandoz, A G, Basel, Switzerland *Ethylenediamine tetra-acetic acid disodium salt (EDTA 2Na)*, Titriplex III, Merck, A G, Darmstadt, Germany *Hog pancreas kallikrein*, padutin R® in ampoules of 10 biol units, Bayer, A G, Leverkusen Germany *Lima bean trypsin inhibitor (LBTI)*, Ovomucoid (OM) and *Soy bean trypsin inhibitor (SBTI)*, Worthington Biochemical Corp, Freehold, New Jersey, U S A

Bioassay The kinin determinations were carried out on the isolated rat uterus as 'bracketing assay' with a dose ratio of 3 2 Bradykinin was used as standard substance

Comments on the Technique

Significance of the acetone concentration for the activity of HP kall preparations against HP kin 2 When prekallikrein in human citrated plasma was activated by incubation with 16 7 % (v/v) acetone, the preparations obtained were only capable of releasing kinin from about 1/3 of the kininogen present in human citrated EDTA plasma and corresponding to the kininogen 1 fractions (BRISEID *et al* 1968, BRISEID *et al* 1973) Even if kininogenase inhibitors were inactivated by heating the kininogen preparation for one hour at 60°, the kinin released during short incubation periods (10-15 minutes) did not significantly exceed the kininogen 1 fractions Table 1, experiment I, demonstrates the rapid activation by hog pancreas kallikrein (padutin) of the kininogen present in HP kin 2 and HP kin 2/60° and also the slow activation caused by a HP kall/17% preparation About 24 hours was required for total kinin release The observation of what was apparently a *50% release by padutin in HP kin 2/60° probably reflected the loss of kal*lidin transforming peptidases during the heating period In the present study activation of prekallikrein with acetone concentrations higher than the 17 % (v/v) standard concentration previously adopted, yielded enzyme preparations more active against HP kin 2 (table 1, experiments II, III) When acetone concentrations of 25% (v/v) or more were used, unknown substances present in the enzyme preparations regularly interfered with the bio assay, potentiating the effect of kinin on the rat uterus By carrying out the activation in the presence of lima bean trypsin inhibitor (LBTI) these difficulties were considerably reduced At the same time the enzyme activity was significantly increased (table 1, experiments II III IV)

Significance of the EDTA treatment for the activities of HP kall preparations

BRISEID *et al* (1973) observed that EDTA 2Na protected the kininogen fraction 1 of human citrated plasma against activation by a human urine kininogenase preparation In the present work we found that the activities and also the stabilities of HP kall preparations were markedly dependent on the point of time of EDTA treatment – before or after the evaporation of acetone According to the original procedure (BRISEID *et al* 1968) the enzyme preparation (HP kall/17%) was incubated with EDTA 2Na for 30 minutes at 37° *after* the evaporation of acetone Fig 1, curve A, shows that such a procedure yielded an enzyme preparation highly active against the kininogen 1 fractions The activity, however, decreased rather quickly When EDTA 2Na was added *before* the

Table 1

Determination of kinin release in human plasma kininogen preparations by different kininogenase preparations

Enzyme concentrations: HP kall preparations 1 ml per ml plasma
Padutin, 3.2 units per ml plasma
LBTI HP kall/17% and LBTI HP kall/25%: Lima bean trypsin inhibitor 2 mg per ml plasma present during acetone activation
Incubation temperature 37°
Kinin released calculated as bradykinin
For further details see text

Experiment	Incubates	µg kinin per ml plasma	Incubation period
I	HP kin 2+padutin	2.1	30 min
	HP kin 2+HP kall/17%	0.1	30 min
	HP kin 2/60°+padutin	1.1	30 min
	HP kin 2/60°+HP kall/17%	<0.1	15 min
	HP kin 2/60°+HP kall/17%	0.1	30 min
	HP kin 2/60°+HP kall/17%	0.6	60 min
	HP kin 2/60°+HP kall/17%	1.0	2 hours
	HP kin 2/60°+HP kall/17%	1.6	4 hours
	HP kin 2/60°+HP kall/17%	1.9	24 hours
II	HP kin 2/60°+HP kall/17%	0.4	30 min
	HP kin 2/60°+HP kall/17%	0.6	60 min
	HP kin 2/60°+LBTI HP kall/17%	0.5	30 min
	HP kin 2/60°+LBTI HP kall/17%	1.0	60 min
III	HP kin 2/60°+HP kall/25%	0.9	30 min
	HP kin 2/60°+LBTI HP kall/25%	1.9	30 min
IV	HP kin 2/60°+LBTI HP kall/17%	0.9	30 min
	HP kin 2/60°+LBTI HP kall/17%	1.2	60 min
	HP kin 2/60°+LBTI HP kall/25%	1.9	30 min
	HP kin 2/60°+LBTI HP kall/25%	2.2	60 min

evaporation of acetone the immediate activity against the kininogen 1 fractions was somewhat lower but more stable (fig. 1, curve C). The activity against the kininogen 2 fraction was low for both EDTA treatment procedures (fig. 1, curves B and D).

Fig. 2 demonstrates for both HP kall/25% and HP kall/17% how the treatment with EDTA 2Na influenced the kininogenase activity against the kininogen 2 fraction. This experiment differed from the experiment shown in fig. 1 by the presence of LBTI during the activation of the enzyme preparations. It can be seen that incubation with EDTA *after* the evaporation of acetone yielded a highly active and stable HP kall/25% preparation and a weak and stable HP kall/17% preparation (fig. 2, curves A and D). Incubation with EDTA *before* the evaporation of acetone yielded a HP kall/25% preparation with a high initial activity but the activity decreased rapidly during the *first* 3 hours

Curve A and C Release of kinin from kininogen fractions 1
Human plasma kininogen (HP kin/60°) incubated with HP kall/17 % (1+1 v/v) for 15 minutes at 37°
Curves B and D Release of kinin from kininogen fraction 2
Human plasma kininogen 2 (HP kin 2/60°) incubated with HP kall/17 % (1+1 v/v) for 30 minutes at 37°
Curves A and B Incubation of HP kall/17 % with EDTA 2Na for different periods of time *after* the evaporation of acetone
Curves C and D Incubation of HP kall/17 % with EDTA 2Na for different periods of time *before* the evaporation of acetone
Kinin released calculated as μg bradykinin/ml plasma
For further details see text

Fig 1 *Significance of the EDTA treatment for the activity of human plasma kallikrein (HP kall/17 %) against kininogen fractions 1 and 2*

and then more slowly (fig 2 curve C) The initial activity of HP kall/17% was rather low but rose to a higher level during the first 3 hours before slowly decreasing (fig 2 curve B)

Effect of temperature on the release of kinin from kininogen fractions 1 and 2

With the use of HP kin 2/60° it was possible to determine kininogenase activity on the basis of kininogen fraction 2 without interference from fractions 1 The estimation of kininogenase activity based on the release of kinin from the kininogen fractions 1 in HP kin/60°, however, was complicated by some release from the kininogen fraction 2 and even for short incubation periods (Briseid *et al* 1973) This interference was increased when the highly active kallikrein preparations obtained by activation with 25% acetone replaced preparations activated by 17% acetone

A selective estimation of kininogenase activity based on the kininogen 1 fractions was made possible however by carrying out the incubation at 0° Fig 3, curve A shows that the rate of release of kinin from HP kin 2/60° was too slow at this temperature to interfere significantly with an assay based on the release of kinin from the kininogen 1 fractions of HP kin/60° (fig 3 curve B) By incubating HP kin/60° at 0° for 30 minutes only a very small amount of kinin is released from kininogen 2 It should be mentioned that curve B showed a sharp bend at about 10 minutes possibly reflecting different rates of release of kinin from the 2 kininogen 1 fractions The rate of release of

Human plasma kininogen 2 (HP kin 2/60°) was incubated with the HP kall preparations for 30 minutes at 37° (1+1 v/v)
Curves A and C HP kall/25 %
Curves B and D HP kall/17 %
Curves A and D Incubation of HP kall with EDTA 2Na for different periods of time *after* the evaporation of acetone
Curves B and C Incubation of HP kall with EDTA 2Na for different periods of time *before* the evaporation of acetone
Kinin released calculated as μg bradykinin/ml plasma
For further details see text

Fig 2 *Significance of the EDTA treatment for the activities of preparations of human plasma kallikrein (HP kall/25 % and HP kall/17 %) against kininogen fraction 2*

Curve A Incubation at 0° of human plasma kininogen 2 (HP-kin 2/60°) with HP kall/25 % (1+1 v/v)
Curve B Incubation at 0° and then 37° of human plasma kininogen (HP kin/60°) with HP kall/25 % (1+1 v/v)
Kinin released calculated as μg bradykinin/ml plasma
For further details see text

Fig 3 *Significance of the temperature for the rate of release of kinin from different kininogen fractions in human citrated plasma*

kinin after about 1 hour corresponded to the rate of release of kinin from HP kin 2/60° (fig 3 curve A) When the temperature was raised from 0° to 37° after 4 hours, the remaining amount of the kininogen 2 fraction was rapidly activated (fig 3, curve B)

Fig 4 shows concentration effect curves for HP kin/60° and HP kall/25% at 37° and at 0° (curves A and B) and also for HP kin 2/60° and HP kall/25% at 37° (curve C) With a release of kinin at 0° of about 0 7 μg per ml HP kin/60° and corresponding to about 0 18 ml HP kall/25% in fig 4 curve B the concentration effect curve flattened out probably indicating a much slower rate of release at 0° of kinin from one of the two kininogen 1 fractions

Results

Effects of protease inhibitors on the kinin release

Table 2, experiments I and II, shows the results of experiments carried out with HP kall/17 % pretreated with different protease inhibitors LBTI and ovomucoid did not significantly interfere with the kinin release, while SBTI partly inhibited the release process The inhibition of the kininogenase activity directed against the HP kin 1 fractions of HP kin/60° was influenced roughly to the same extent as was the kininogenase activity directed against the HP-kin 2/60° preparation tested The two inhibitor experiments accordingly did not differentiate between kininogenase activities Experiment *III* in table 2 shows that HP-kall/17 % heated at 60° for one hour was still capable of causing some kinin release in 60°-heated HP kin 2, and that this release was not significantly inhibited by SBTI

Plasma kallikrein preparation HP kall/25 %
Curve A Substrate HP kin/60° kininogen 1
Incubation 10 minutes at 37°
Curve B Substrate HP kin/60° kininogen 1
Incubation 30 minutes at 0°
Curve C Substrate HP kin 2/60° kininogen 2
Incubation 30 minutes at 37°
Kinin released calculated as μg bradykinin/ml plasma
For further details see text

Fig 4 *Plasma kallikrein concentration effect curves*

Table 2

Effects of protease inhibitors on the kinin release from 2 kininogen fractions

Enzyme concentration 1 ml per ml kininogen preparation
Incubation temperature 37°
Enzyme inhibitors
Lima bean trypsin inhibitor (LBTI), 2 mg/ml HP kall
Ovomucoid (OM) 10 mg/ml HP kall
Soy bean trypsin inhibitor (SBTI), 1 mg/ml HP kall
HP kall pre incubated with inhibitors for 10 minutes at 37°
Kinin released was calculated as bradykinin
For further details see text

Experiment	Incubates		μg kinin per ml plasma	Incubation period
I	HP kin/60°+	HP kall/17 %	1 4	15 min
	HP kin/60°+LBTI	HP kall/17 %	1 5	15 min
	HP kin/60°+OM	HP kall/17 %	1 4	15 min
	HP kin/60°+SBTI	HP kall/17 %	0 5	15 min
II	HP kin 2/60°+	HP kall/17 %	0 7	60 min
	HP kin 2/60°+LBTI	HP kall/17 %	0 6	60 min
	HP kin 2/60°+OM	HP kall/17 %	0 6	60 min
	HP kin 2/60°+SBTI	HP kall/17 %	0 3	60 min
III	HP kin 2/60°+	HP kall/17 %	0 7	60 min
	HP kin 2/60°+	HP kall/17 %/60°	0.3	60 min
	HP kin 2/60°+SBTI	HP kall/17 %/60°	0 3	60 min

Assay of different preparations of HP-kall

Table 3 shows the results of determinations of the kininogenase and esterase activities of 3 different preparations of acetone activated human plasma kallikrein (I, II, III) The activity of the enzyme preparation which proved to be the strongest one (I), was set to 100 %

In the experiment shown in table 3, deviations in the preparation procedures for the enzymes were restricted to (1) different acetone concentrations and (2) different point of time of EDTA treatment In all 3 preparations LBTI (2 mg/ml plasma) was present during acetone activation and the EDTA-contact period was fixed at 5 hours

Table 3 shows that HP kall/25 % with acetone evaporated after the EDTA-treatment (II) was much weaker than HP-kall/25 % with acetone evaporated before EDTA treatment (I) and that the *activity against the* kininogen 1 fraction was lost to the greatest extent (kin 1/kin 2 – ratio 0 5) For HP-kall/17 % the loss of activity was more pronounced when estimated on the basis of the kininogen 2 fraction (kin 1/kin 2 – ratio 1 4). In both

Table 3.

Assay of different preparations of acetone activated human plasma kallikrein

Substrates Kininogen fraction 1 in HP kin/60°
Kininogen fraction 2 in HP kin 2/60°
Benzoyl arginine ethyl ester (BAEe)
Incubation periods Kininogen fraction 1, 10 min at 37° 30 minutes at 0°
Kininogen fraction 2, 30 min at 37°, 4 hours at 0°
Kinin released tested on the isolated rat uterus and calculated as bradykinin
For further details see text

	Enzyme preparation (After acetone activation EDTA treatment 5 hours at 37°)	Activities in per cent at different substrates							Activities $\frac{0°}{37°}$	
		37°			25°	0°				
		Kin 1	Kin 2	$\frac{\text{Kin 1}}{\text{Kin 2}}$	BAEe	Kin 1	Kin 2	$\frac{\text{Kin 1}}{\text{Kin 2}}$	Kin 1	Kin 2
I	HP kall/25 % Acetone evap before EDTA	100	100	1 0	100	100	100	1 0	1 0	1 0
II	HP kall/25 % Acetone evap after EDTA	12 5	25	0.5	27	7	15	0 5	0.56	0 60
III	HP kall/17 % Acetone evap after EDTA	50	36	1 4	59	40	30	1.3	0 80	0 83

HP kall preparation I and II the esterolytic activity was close to or somewhat higher than the highest kininogenase activity recorded irrespective of the kininogen fraction

It can be seen from table 3 that the activities of the kallikrein preparations II and III when compared with preparation I were significantly lower at 0° than at 37° The kin 1/kin 2 – ratios, however, were the same at 0° as those observed at 37°, indicating that the kininogenase activities tested were the same at the two temperatures

Discussion

The observation that the relative activities of different preparations of acetone-activated human plasma kallikrein varied with the substrates used, kininogen 1 and kininogen 2, might reflect the presence of more than one kininogenase in the enzyme preparations The results might, however, also be due to kininogenase inhibitors or other interfering substances being present in the crude enzyme preparations to a varying extent Purification of the kallikrein together with parallel running quantitative determinations based on the 2 kininogen fractions should be carried out to solve the problem The fact that the relative activities of the enzyme preparations tested were not the same at 0° as those registered at 37°, might indicate an inhibition at 0° of kininogenase inhibitors present in the preparations in different amounts According to LAAKE *et al* (unpublished results) both α-2-macroglobulin and CI inactivator are only slightly active against human plasma kallikrein at 0° The data accordingly demonstrate that different levels of temperature-sensitive kininogenase inhibitors were responsible for the different kininogen 1/kininogen 2 activity ratios observed

REFERENCES

Briseid K. O K Dyrud & F C Arntzen Determination of prekallikrein in human plasma. *Acta pharmacol et toxicol* 1968 26 395–401

Briseid K. O K Dyrud & F C Arntzen Substrates for kininreleasing enzymes in rat plasma *Acta pharmacol et toxicol* 1970 28 138–144

Briseid K A M Vennerod & E Andrew Substrates for kinin releasing enzymes in human plasma *Acta pharmacol et toxicol* 1973 32 193–204

Acta pharmacol et toxicol 1973, 32, 214–224

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Anticholinergic and Anti-arrhythmic Effects of Phenoxybenzamine on the Isolated Rat Atrium

By

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Abstract The effect of phenoxybenzamine, an α adrenergic receptor blocking agent belonging to the β haloalkylamine group of drugs has been tested on isolated rat atria Phenoxybenzamine in concentrations ranging from 5×10^{-6}M to 5×10^{-5}M had positive chronotropic, but negative inotropic effects At higher dose levels (10^{-4}M and 5×10^{-4}M) the rate decreased whereas the depressing effect on contractile force was almost unchanged The threshold for electrical stimulation and the effective refractory period were not affected Phenoxybenzamine 2.5×10^{-5}M protected against the changes in the rate and force of atrial contractions caused by acetylcholine 5×10^{-6} M In addition the changes in the rat electrogram induced by acetylcholine were prevented by the drug Atrial flutter and fibrillation produced by a standardized combination of electrical stimulation and acetylcholine were converted into sinus rhythm after the addition of phenoxybenzamine 7.5×10^{-5}M

Key words Phenoxybenzamine – rat atria – anticholinergic effect – anti arrhythmic effect

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Anti arrhythmic activity of dibenamine, an α-adrenergic blocking agent belonging to the β-haloalkylamine group of drugs has been described previously In dogs, the compound was found to prevent cyclopropane-adrenaline-induced cardiac arrhythmias (NICKERSON & NOMAGUCHI 1949), and in man, preoperative treatment with dibenamine almost completely eliminated cyclopropane-induced ventricular arrhythmias (NICKERSON & BROWN 1951) In addition, MALING & HIGHMAN (1958), reported that phenoxybenzamine (bensylytum NFN), another β-haloalkylamine drug prevented nor-adrenaline induced ventricular tachycardia in dogs

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The mechanisms underlying the anti arrhythmic activity of β-haloalkylamine drugs have not yet been clarified It has previously been suggested that the mode of action of the anti arrhythmic activity of dibenamine includes a direct effect on the myocardium prevention of the adrenaline-induced rise in systolic blood pressure or prevention of reflex vagal activity by abolishing the pressor response to adrenaline (NICKERSON & NOMAGUCHI 1949) A quinidine like action of the drug has been suggested (GARB & CHENOWETH 1948) However, as phenoxybenzamine is assumed to be devoid of local anaesthetic activity (IVERSEN 1971), the anti arrhythmic activity of this drug and of dibenamine is probably not caused by a quinidine-like effect Phenoxybenzamine is a blocking agent for α adrenergic receptors but is also capable of inhibiting the response to a wide variety of other agents, including acetylcholine (Ach) (COOK 1971) In the isolated guinea pig atrium, the drug abolished the effects of vagus stimulation (BENFEY & GREEFF 1961) The question therefore arises whether vagus-overactivity might be involved in cases of arrhythmias prevented by β haloalkyl amines The vagal component in adrenaline-induced arrhythmias has previously been stressed (WILBURNE *et al* 1947), and it has been shown that Ach can produce cardiac arrhythmias *in vitro* (BURN *et al* 1955, HOLLAND & BURN 1957, HOLLAND & BRIGGS 1959)

The present study was undertaken in an attempt to elucidate the mode of action of phenoxybenzamine on isolated rat atria, and the purpose was primarily to see if the drug was capable of blocking the effect of Ach, and also abolishing Ach induced fibrillation of isolated atria

Methods

Female albino Wistar rats weighing about 200 g were used The procedures used in the isolation of rat atrial muscles and the recording techniques have been described previously (REFSUM & LANDMARK 1972) The Ringer solution which was kept at 32°, had the following composition (ions in meq./l) Na^+ 143 4 K^+ 5 3 Ca^{++} 5 1 Mg^{++} 2 3 Cl 126 4 $H_2PO_4^-$ 2 4 HCO_3^- 25 SO_4^{--} 2 3 glucose 1 8 mg/ml and the solution was continuously bubbled with 95% O_2 and 5% CO_2 The pH was 7 4

When the atria were electrically driven this was done through bipolar platinum electrodes by square wave pulses of 0 5 msec duration delivered by a Biotronic laboratory stimulator The intensity of the stimulus was measured on a Tektronix dual beam oscilloscope type 502A coupled in series with the atria To record atrial electrograms one platinum electrode was placed at the apex of the left atrium while a reference platinum electrode was placed in the organ bath The electrogram and the isometric contraction were simultaneously recorded by the Grass polygraph Control experiments were performed at the end of a 30 min period of equilibration

Atrial rate and contractile force

To test the effect of phenoxybenzamine on the atria the drug was added to the organ bath in concentrations ranging from 5×10^{-6}M to 5×10^{-4}M Left and right spontaneously beating atria were used The effect of each was measured over 8 min. after the addition of the drug Then the bathing solution was changed twice When stable values approximately like the control values were obtained, a new addition was made with a higher concentration of phenoxybenzamine Log dose response curves were calculated for the inotropic and chronotropic effects of the drug

Electrical threshold and maximum following frequency

In order to measure the threshold values required to drive the atria, the stimulus frequency was set about 10 per cent higher than the spontaneous atrial rate measured at the end of the equilibration period The intensity of the stimulus was then gradually increased until the atria followed the imposed frequency

When measuring the maximum following frequency (m f f) the stimulus intensity required to drive the atria was doubled The rate of the stimulation was increased until the atria failed to follow, i e when they began to drop beats The duration of the pulses was kept constant

Anticholinergic effect

Left and right spontaneously beating atria were used At the end of the equilibration period, the atrial rate and contractile force and the atrial electrogram were recorded Phenoxybenzamine $2 5 \times 10^{-5}$M was then added After 8 min new recordings were made before acetylcholine (Ach) 5×10^{-6} M was added to the organ bath the effect of which was recorded after 2 min Similar experiments were performed at the same time intervals without the addition of phenoxybenzamine

Anti arrhythmic effect

Ach induced atrial fibrillation was produced by making some minor modifications of a method described by HOLLAND & BURN (1957) In these experiments the potassium concentration of the Ringer solution was 1 325 meq./l The composition of the Ringer solution was otherwise as described above Left and right atria were beating spontaneously for 30 min before the atrial rate and contractile force as well as the atrial electrogram were recorded The atria were then stimulated by square wave pulses of 0.5 msec duration at a frequency of 2400 per min for 2½ min The intensity of the stimulus was 6V Ach $2 5 \times 10^{-5}$ M was added to the organ bath 1 min after the stimulation was started The contractions became quite incoordinated during this procedure The atrial contractions and electrograms were recorded when the stimulation was stopped after which phenoxybenzamine $7 5 \times 10^{-5}$M was added Ten min later the atrial rate and contractile force and the atrial electrogram were recorded Similar experiments were performed with the administration of deionized water instead of phenoxybenzamine

Calculations

In order to compare the results, the values (for force and rate of concentrations) measured at the end of the equilibration period in each experiment were set at 100 per cent and used as reference Comparison of the results was made by using the Student s test

Drugs used

Phenoxybenzamine HCl (dibenzyline) (Smith Kline and French Labs Ltd Herts England) and acetylcholine chloride in deionized water

Results

Effect of phenoxybenzamine on atrial rate and contractile force

The atria resumed spontaneous contractions immediately after they had been placed in the organ bath The rate of the atrial contractions usually increased during the first 2–3 min , after which it almost remained unchanged throughout the equilibration period of 30 min The contractile force gradually increased until a steady state was reached after about 15 min The addition of phenoxybenzamine caused an increase in the frequency of contractions in the dose range from 5×10^{-6}M to 5×10^{-5}M At higher dose levels (i e 10^{-4}M and 5×10^{-4}M) there was a decrease in the frequency of contractions The administration of phenoxybenzamine had little effect on the atrial contractile force (fig 1)

Among the 7 experiments carried out atrial arrest occurred in one experiment 7 min after the addition of phenoxybenzamine 10^{-4}M, and in another experiment atrial arrest occurred 6 min after the addition of phenoxybenz amine 5×10^{-4}M The atrial rate in the control period varied from 175 to 330 beats per min , and the atrial contractile force from 290 to 940 mg

Effect of phenoxybenzamine on the electrical threshold and maximum following frequency

The threshold for electrical stimulation varied at the end of the control period between 3 and 17 mA Phenoxybenzamine in concentrations between

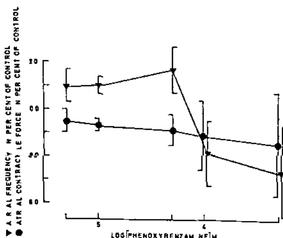


Fig 1 Log-dose response curves showing the inotropic and chronotropic effects of phenoxybenzamine The values (mean value ± S E M) represent the mean of 7 experiments and are calculated as a percentage of the control values obtained before each new addition of the drug

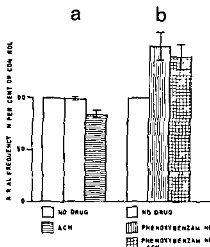


Fig 2 The negative chronotropic effect of Ach 5×10^{-6}M in the absence (a) and presence (b) of phenoxybenzamine $2\,5 \times 10^{-5}$ M The values (mean value ± S E M) represent the mean of 6 experiments and are calculated as a percentage of the values obtained at the end of the control period (left white columns in a and b) The difference in the response to Ach in the absence or presence of the drug is statistically significant ($0\,005 < P < 0\,01$)

5×10^{-6} and 5×10^{-4} M did not change these values The addition of phenoxy benzamine $7\,5 \times 10^{-5}$ M did not alter the maximum following frequency to any significant extent

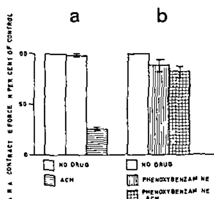


Fig 3 The negative inotropic effect of Ach 5×10^{-6}M in the absence (a) and presence (b) of phenoxybenzamine $2\,5 \times 10^{-5}$M The values (mean value ± S E M) represent the mean of 6 experiments and are calculated as a percentage of the values obtained at the end of the control period (left white columns in a and b) The difference in the response to Ach in the absence or presence of the drug is statistically significant ($P < 0\,001$)

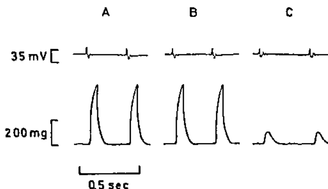


Fig 4 Atrial electrograms and contractions at the end of the control period (A), 8 min later (B) and 2 min after the addition of Ach 5×10^{-6} M (C)

Anticholinergic effect of phenoxybenzamine

Ach 5×10^{-6} M caused both a decrease in the force and the rate of the contractions, the latter effect being less pronounced than the former Phenoxybenzamine 2.5×10^{-5} M blocked these effects of Ach (figs 2 and 3) As shown in fig 4, Ach 5×10^{-6} M caused marked changes in the atrial electrogram However, when phenoxybenzamine 2.5×10^{-5} M was added before the addition of Ach 5×10^{-6} M, no obvious changes occurred (fig 5) Phenoxybenzamine alone did not alter the atrial electrogram

Anti-arrhythmic effect of phenoxybenzamine

The combination of electrical stimulation and Ach 2.5×10^{-5} M caused a quite incoordinated atrial rhythm At the same time, the atrial electrograms showed fibrillation or flutter (fig 6) Phenoxybenzamine 7.5×10^{-5} M con-

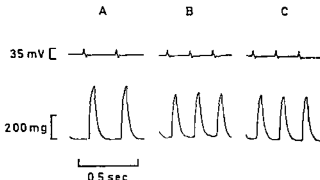


Fig 5 Atrial electrograms and contractions at the end of the control period (A) 8 min after the addition of phenoxybenzamine 2.5×10^{-5} M (B) and 2 min after the subsequent addition of Ach 5×10^{-6} M (C)

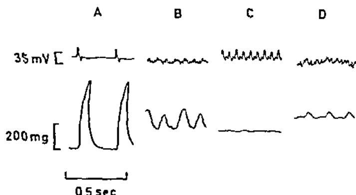


Fig 6 Atrial electrograms and contractions at the end of the control period (A) and examples of atrial fibrillation (B and D) and atrial flutter (C) throughout the test period.

verted these incoordinated contractions into regular contractions 3 8 ± 0 4 min. after the addition of the drug (fig 7) In 5 control experiments in which no drug was added, the atria continued to fibrillate throughout the whole test period

Discussion

The results presented in this study show, that phenoxybenzamine in concentrations between 5×10^{-6} M and 5×10^{-5} M had positive chronotropic and slight negative inotropic effects in spontaneously beating rat atria At higher dose levels the mean rate decreased whereas the negative inotropic effect did not increase further The threshold for electrical stimulation was unaltered at all dose levels used The determination of maximum following frequency has been widely used as a screening standard for the effective refractory

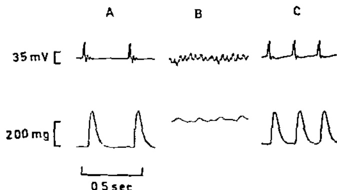


Fig 7 Atrial electrograms and contractions at the end of the control period (A) atrial fibrillation (B) and atrial electrograms and contractions 10 min after the addition of phenoxybenzamine 7.5×10^{-5} M (C)

period (REFSUM & LANDMARK 1972) As this parameter was not influenced by phenoxybenzamine to any significant extent, it shows that this drug has no effect on the effective refractory period These results indicate that phenoxybenzamine has no effect on the electrical activity of rat atria, which is further confirmed by the absence of effect of phenoxybenzamine on atrial electrograms This supports the assumption that the drug in the concentrations used in these experiments, possesses no local anaesthetic or quinidine-like activity which could explain the anti arrhythmic effect of the drug The possibility therefore exists that arrhythmias prevented by phenoxybenzamine are caused by direct or indirect sympathetic-parasympathetic interactions in the heart The experiments show that phenoxybenzamine is capable of blocking the effects of Ach on the atrial rate and contractile force, and also of preventing the changes in the atrial electrogram caused by this transmitter The results are consistent with those obtained by BENTLEY & GRILLO (1963), who observed an atropine like antagonism between Ach and phenoxybenzamine

The β haloalkylamine drugs do not block the stimulatory effects of sympaticomimetic amines on the heart (FURCHGOTT 1959, NICKERSON & CHAN 1961, MORAN & PERKINS 1961) On the contrary, it has been demonstrated that phenoxybenzamine increases the changes in the rate and force of isolated atria caused by adrenaline and noradrenaline (STAFFORD 1963) and increases the force of contractions of electrically stimulated rat ventricles produced by noradrenaline (FARRANT *et al* 1964) In isolated frog ventricular muscle, the drug potentiated the response to adrenaline on the action potential (MCCALLUM & RODDIE 1964) Finally, it has been shown that treatment with phenoxybenzamine leads to increased synthesis and release of noradrenaline in the heart of rats (DAIRMAN *et al* 1968) It is therefore unlikely that arrhythmias prevented by β haloalkylamines are entirely due to stimulation of β receptors N isopropyladrenaline, which has a powerful action on β-receptors and almost no action on α receptors, is in contrast to adrenaline and noradrenaline, not capable of inducing ventricular fibrillation in cats during hydrocarbon inhalation (GARB & CHENOWETH 1948) *Adrenaline increases the rate in isolated heart preparations, whereas* in the intact, unanaesthetized animal, a slowing of the heart rate occurs, often in combination with various cardiac dysrhythmias, due to a reflex from the carotid sinus and the aortic depressor mechanism acting through the vagi (WILBURNE *et al* 1947) Atropine abolishes these changes Thus, it is possible that cyclopropane adrenaline induced cardiac arrhythmias are due to a peripheral α-adrenergic stimulation in the arteries, followed by a stimulation of the vagi Vagal stimulation or parasympaticomimetic drugs can precipitate arrhythmias, or increase the rate of an established auricular fibrillation (SCHERF & SCHOTT 1953, BURN *et al* 1955) During vagal stimula-

Refsum, H & K Landmark The action of practolol on the isolated rat atrium *Acta pharmacol et toxicol* 1972, **31**, 97–106

Scherf, D & A Schott *Extrasystoles and allied arrhythmias* Heineman, London, 1953

Sharma P S Mechanisms of atrial flutter and fibrillation induced by aconitine in the dog, with observations on the role of cholinergic factors *Brit J Pharmacol* 1963, **31**, 368–377

Stafford, A Potentiation of some catecholamines by phenoxybenzamine, guanethidine and cocaine *Brit J Pharmacol* 1963, **21**, 361–367

Trautwein, W Generation and conduction of impulses in the heart as effected by drugs *Pharmacol Rev* 1963, **15**, 277–332

Wilburne, M, A Surtshin, S Rodbard & L N Katz Inhibition of paroxysmal ventricular tachycardia by atropine *Amer Heart J* 1947, **34**, 860–870

Acta pharmacol et toxicol 1973, 32, 225–236

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Uptake and Retention of the Enantiomers of a Local Anaesthetic in Isolated Nerve in Relation to Different Degrees of Blocking of Nervous Conduction

By

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Abstract Sciatic nerves of the frog were soaked with equimolar solutions of the ^{3}H-enantiomers of compound RAC 109, a spirosuccinimide with local anaesthetic action The enantiomers were taken up equally by (1) sheathed nerves during 2 hrs soaking with 2 5 mM solutions (2) sheathed nerves and 10 min soaking with 1.25–40 0 mM solutions and (3) desheathed nerves during 1 hr soaking with 0 25 mM solutions The washout curves for the two forms after application (2 5 mM) to sheathed nerves for 1 hr were identical Recording of the block of the action potential with simultaneous determination of the concentration of the compound in sheathed nerve revealed a difference in the rate of block (5 0 mM) and the depth of block at equilibrium (2 5 mM) between the enantiomers but the same uptake and retention in nerve The epineural sheath was not a stereo-selective barrier to penetration Series of equilibrium blocks with 0 01–0 4 mM ^{3}H enantiomers showed an approximately four fold difference in concentration in de sheathed nerve with a 50 per cent block of nervous conduction The enantiomer RAC 109 I was consistently more potent than RAC 109 II The findings suggest that the difference in the nerve blocking potency of the enantiomers can hardly be ascribed to a difference in the uptake of the compounds by nerve

Key words Local anaesthetics – enantiomers – optical isomers

Enantiomers of some local anaesthetics differ in their nerve blocking potency both *in vivo* and *in vitro* ÅKERMAN *et al* (1967b & 1969), for example, found that in a series of aminoalkyl-tetralin spirosuccinimides one enantiomer was always more potent in various excitation block tests *in vitro* The maximum potency ratio was about eight Similar results were obtained with enantiomers of aminoacylephedrines (SCHONENBERGER *et al* 1967a & b) and carbanilic acid esters of cyclic amino alcohols (ÅKERMAN 1972) The findings of these studies with three different types of compound suggest some

stereo-selectivity in the nerve blocking action of local anaesthetics However even in an *in vitro* system like the isolated nerve, the possibility remains that enantiomers are distributed differently thus to resulting in differences in concentration of the two forms in the biophase One possible method of elucidating this problem would be to study the relationship between nerve blocking potency *in vitro* and drug uptake A study of this kind was therefore done with the enantiomers of one of the aminoalkyl tetralin spirosuccinimides tested earlier (AKERMAN *et al* 1969) The uptake of ^{3}H-compound into sheathed and de sheathed sciatic nerve of the frog was determined both with and without simultaneous recording of nervous conduction blocks

Material and methods

Compound RAC 109 (N (α-diethylaminopropyl) 1 2 3 4-tetrahydronaphthalene 1 spiro succinimide) belongs to a new series of local anaesthetics (SANDBERG 1967) The enantiomers of the imide were prepared (SANDBERG unpublished results) from 1 carboxy 1 tetralinacetic acid resolved by means of cinchonidine and strychnine The relative configuration was correlated by chemical interconversion The steric relationship is indicated by denoting the appropriate enantiomer RAC 109 I or RAC 109 II The enantiomers had optical rotations of the same size but opposite sign ^{3}H labelling of the enantiomers was carried out according to the method of TELC *et al* (1971) The specific activity of ^{3}H RAC 109 I and ^{3}H RAC 109 II was 26 0 μci/mg and 22 5 μci/mg respectively The melting point of the enantiomers before and after labelling was identical (169–170°) Solutions with the same activity (2.5 μci/mg) were used and prepared by adding a non labelled drug to the labelled material The enantiomers were used as hydrochlorides and dissolved in Tasaki Ringer (111 2 mM NaCl 1 88 mM KCl 1 0 mM CaCl 2 38 mM $NaHCO_3$ and 0 06 mM NaH_2PO_4)

Determinations of drug uptake and efflux in sheathed and desheathed nerve were made with frog (*Rana Pipiens*) sciatic nerves The nerves were removed by dissection and freed from accompanying fascia Branches of the nerve – with the exception of the peroneal branch were cut a few millimetres from their bifurcation points Nerves free of the epineural sheath were prepared under a dissection microscope the de sheathing being started at a proximal site of bifurcation During preparation and before the experiment the nerves were soaked in Tasaki Ringer solution pH 7 0 After preparation series of four nerves ligated at both ends were attached to a plexiglass holder and suspended for various periods of time in a beaker containing the radioactive anaesthetic agent in 50 ml Tasaki Ringer solution The same solution without the local anaesthetic was used for the washout experiments in which superfusion was performed at a constant rate (16 7 ml/min) At the end of the bathing or rinsing period the nerves were cut free from ligatures quickly blotted on filter paper and weighed A slight progressive swelling and increase in weight of the nerves was observed and was independent of the type of anaesthetic solution used The weighed nerves and in one trial the epineural sheaths were disintegrated in 1 0 ml of Soluene 100TM (Packard) and 50 μl of aq dest for 24 hours in a 37° water bath Fifteen ml of scintillation liquid containing 0 4 % 3 4 diphenyloxazole and 0 01 % β bis [2 (phenyloxazolyl)] benzene in toluene *(9/10) and* ethanol (1/10) were added before measuring the radioactivity Standards were made by

adding known amounts of the labelled compounds to the disintegrating fluid and treating the samples in the same way as the test samples It was recognized that surface adsorption may to some extent influence the uptake values particularly in the experiments with short exposure times However, the surface adsorption values obtained after rapidly dipping the nerve in the active solution did not differ for the two enantiomers

The method described by TRUANT (1957) and ÅSTRÖM & PERSSON (1961) was used for the recording of block of action potential For the rate block experiments a 1.5 cm portion of the isolated nerve between the stimulating and recording electrodes was immersed in a 25 ml bath with the test solution contained in a moist chamber at 20° The stimulus (0 05 msec pulses at frequencies of 30 pulses/sec 2 5 V) was provided by a Grass stimulator (S4E) coupled with a stimulus isolation unit The action potential was monitored on a Textronic oscilloscope (502) and the depression of the A spike recorded against time The spike amplitude was calculated as a per cent of the initial spike amplitude In other experiments e g the equilibrium blocks the whole nerve was exposed to the test solution The amplitude of the action potential was measured in air at regular intervals during soaking with the test solution and/or rinsing with Tasaki Ringer Washing out with Tasaki Ringer was performed at a constant rate (16 7 ml/min) The effects of the enantiomers were compared on paired nerves The amount of compound in the nerve was determined at the end of the experiment in the same manner as described above

The Student s t test was used to test the statistical significance A significant difference had a P value of $\leq$ 0 05 Line segments of log dose response curves were calculated according to the formula $y=a+b\log x$ on an Olivetti Programma 101

Results

Determination of drug uptake and efflux in sheathed and de-sheathed nerve

The uptake of the enantiomers by sheathed nerves in a 2 5 mM solution was studied With both enantiomers a 2 5 mM solution is below the threshold concentration for the blocking of conduction in sheathed nerves (see page 228) As seen in fig 1A there was a more rapid initial uptake phase with both compounds, followed by a slower uptake Equilibrium was not reached during a soaking period of 120 minutes Although slightly more of enantiomer 109 II was taken up at all the times studied except at 5 minutes, the difference never reached the level of statistical significance (P $\leq$ 0 05)

The results of a study with de sheathed nerves are illustrated in fig 1B Using 0 25 mM solutions a rapid initial uptake occurred followed by a slower uptake phase Equilibrium was not reached after a bathing period of 60 minutes As with sheathed nerves there was no difference in the accumulation of the enantiomers by de sheathed nerves

In another series of experiments sheathed nerves were bathed for 10 minutes in 1 25–40 0 mM solutions of the compounds and the uptakes determined As seen in fig 2 there was no difference between the enantiomers in the amounts taken up at the concentrations used. Large amounts of the

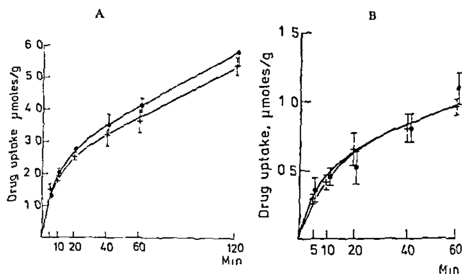


Fig 1 Uptake of ^{3}H RAC 109 I (+) and ^{3}H RAC 109 II (●) by sheathed (1A) and de sheathed (1B) sciatic nerve of the frog Each point is a mean obtained from 6 nerves ± S E M 2.5 mM solutions were used on sheathed nerve and 0 25 mM solutions on sheathless nerve, pH 7 0

compounds accumulated in the nerve trunk without any signs of saturation during the 10 minute period

The efflux of the enantiomers was studied on sheathed nerves The nerve trunks were soaked in 2 5 mM solutions for 60 minutes and then washed

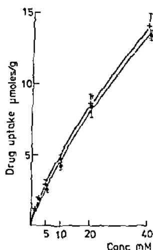


Fig 2 Uptake of the ^{3}H enantiomers of RAC 109 by sheathed sciatic nerve of the frog after a soaking period of 10 minutes using varying concentrations pH 7 0 Each point is the mean ± S E M obtained from 6 nerves (+) RAC 109 I (●) RAC 109 II

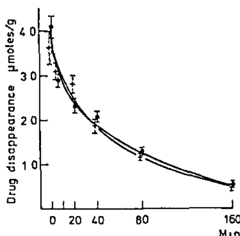


Fig 3 Efflux of ³H RAC 109 I (+) and ³H RAC 109 II (●) from sheathed sciatic nerve of the frog 2.5 mM solutions pH 7 0 Mean ± S E M from 6 nerves

by superfusion with Tasaki-Ringer solution Fifty per cent of the amount taken up remained in the tissue after about 40 minutes of washing (fig 3) Washout over a period of 160 minutes did not result in complete disappearance of the compounds The concentration of the enantiomers in the sheathed nerves at the various times used did not differ significantly

Recording of block of action potential with simultaneous determination of concentration of compound in the nerve

In a first series a segment of a sheathed sciatic nerve between the pairs of stimulating and recording electrodes was soaked in 5 0 mM radioactive solutions of the compounds and the depression of the propagated action potential (A spike) was recorded after various bathing periods The amount of compound in the exposed portion (2 5 cm) of the nerve was determined at the end of the soaking period As seen from fig 4A, both compounds blocked conduction completely at the concentration used However, enantiomer 109 I produced a more rapid block than enantiomer 109 II With the exception of the 2 minute soaking period there was a statistically significant difference between the enantiomers with regard to the depth of block The uptake curves for the two forms (fig 4B) showed that in this series too the enantiomers were taken up equally The accumulation had not reached a steady state after a soaking time of 40 minutes Comparing the curves for the blocking of the action potential and uptake into the nerves, it is obvious that higher amounts of enantiomer II than enantiomer I were needed for the same degree of nerve block With compound RAC 109 II a 50 % block

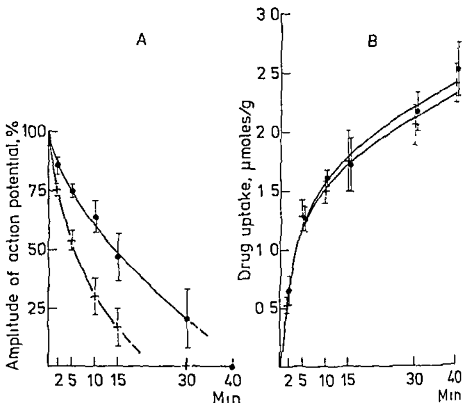


Fig 4 Sheathed sciatic nerve of the frog Block of evoked action potential (A) and accumulation in the nerve (B) upon treatment with 5 0 mM ^{3}H RAC 109 I (+) and ^{3}H RAC 109 II (●), pH 7 4 Each point represents the mean ± S E M depression of action potential (A) and uptake (B) from 4 trials (10 minutes, N = 8)

corresponded to a tissue content of approximately 1 70 µmol/g as compared with about 1 30 µmol/g for enantiomer RAC 109 I

In another series of experiments entire sheathed nerves were soaked in 5 0 mM RAC 109 I and II for 40 minutes and then washed for various periods of time With both enantiomers the compound action potential was completely blocked at the end of the 40 minute soaking period After 40 minutes of treatment the concentration of compound in the nerves was 2 31±0 31 µmol/g with 109 I (N=6) and for 109 II 2 68±0 27 µmol/g (N=9) The difference was not statistically significant The results of subsequent washout experiments are illustrated in fig 5 The recovery from blocking with enantiomer RAC 109 I was much slower than that with RAC 109 II (fig 5A) For example after 80 minutes of washing the block produced by 109 I was 74±8 % as compared with a decrease to a 25±14 %

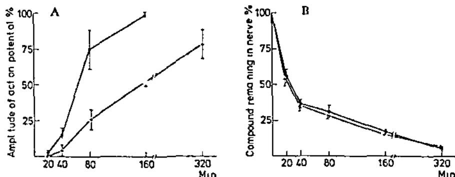


Fig 5 Sheathed sciatic nerve of the frog Recovery from block of conduction (5A) and efflux (5B) of compound on washout after a soaking period of 40 minutes Each point represents the mean ± S E M depression of the action potential and efflux respectively from 4–9 trials with 5 0 mM (pH 7 4) ^{3}H RAC 109 I (+) and II (●)

block with 109 II (0 05 > P > 0 01) However, this difference in duration of action was not related to a difference in the efflux of the agents from the nerve As seen from fig 5B, the concentrations of the two enantiomers in the nerve did not differ at the end of each washing period Consistent with the experiments in which the onset of blocking was studied, the present findings show that more 109 II had to be present in the nerve as compared with 109 I in order to attain the same degree of block The amounts required for a 50 % block after washout from a complete block were about 0 4 μmol/g with 109 I and 0 8 μmol/g with 109 II

In a separate study (table 1) sheathed sciatic nerves were soaked in 2 5 mM solutions with the nerve placed on electrodes and tested as in the previous series An equilibrium state of block occurred after the same period of soaking with the enantiomers However, the depth of block was significantly greater with enantiomer 109 I than with enantiomer 109 II The nerves were taken for the assay of radioactivity when the block had been stable for 10 minutes In this series the epineural sheath was pulled off at the end of the soaking period and treated separately from the remainder of the tissue The amount of the compounds recovered from the de-sheathed nerve did not differ significantly for the two enantiomers There was no selective binding to the connective tissue sheath surrounding the nerve trunk

A third series of experiments was made on de sheathed nerves Groups of entire nerves were soaked in varying concentrations (0 01–0 4 mM) of the enantiomers and the decline of the action potential followed until the block was steady The concentrations in the nerves were determined 10 minutes after the steady state of block had set in Fig. 6 is a plot of the log con-

Table 1

Equilibrium block with 2 5 mM ^3H RAC 109 I and II (pH 7 4) on sheathed sciatic nerve of the frog Each value represents mean ± S E M obtained from 6 nerves

Compound	Time to equilibrium block min	Block of action potential %	Amount in tissue µmol/g	
			Desheathed nerve	Epineural sheath
109 I	22 ± 3	91 ± 3	1 83 ± 0 25	1 11 ± 0 27
109 II	18 ± 3	48 ± 5	1 67 ± 0 16	1 12 ± 0 25
Significance of difference P	N S	P < 0 001	N S	N S

centration of compound in the nerve against the depression of the action potential at equilibrium The results with both enantiomers show good cor relation ($r_{109\ I}=0\ 92$, $r_{109\ II}=0\ 85$) between log concentration of the compound in the nerve and the degree of block However, as evidenced by the shift to the right of the line segment of the curve for RAC 109 II, this agent displayed a lower potency as compared with enantiomer RAC 109 I The concentration of 109 I in nerve at a 50 % block of conduction was about 0 32 µmol/g while the concentration of 109 II was about 1 26 µmol/g

Discussion

The uptake of the enantiomers of compound RAC 109 into sciatic nerve trunks seems to involve at least two processes with different rates of uptake indicating an accumulation in different tissue spaces As pointed out by SHANES & BERMAN (1953), distinguishing between different tissue spaces in a compound nerve and, still more, recognition of different compartments within these spaces meets with technical difficulties The sciatic nerve trunk may be subdivided into three major parts the dense connective tissue nerve sheath, the interstitial tissue and the fibre containing fasciculi If this is true, the results of the separate uptake study on entire nerves without simultaneous recording of block of action potential and the equilibrium block experiments both using 2 5 mM solutions may suggest that the accumulation into the nerve fibre phase takes place mainly during the more rapid, early stage of uptake The late accumulation phase seen in the former experiments may

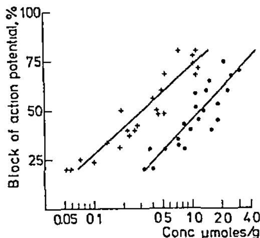


Fig 6 Equilibrium block of conduction in de-sheathed sciatic nerve of the frog in relation to the concentration of the compound in the nerve Series of 4–7 nerves were soaked in 5 different concentrations (0 01–0 4 mM pH 7 4) of ^{3}H RAC 109 I (+) and ^{3}H RAC 109 II (●) The concentration in the nerve was determined after the same period of soaking with the enantiomers and when a stable state of block had occurred Values below 20 and above 80 % block not plotted

reflect uptake mainly in the loose lacework of the interstitial connective tissue which probably accounts for the recorded weight (water) increase with time

Confirming previous results (ÅKERMAN *et al* 1969), the evoked action potential in isolated sciatic nerve of the frog was significantly more depressed by enantiomer RAC 109 I than by RAC 109 II, both with regard to rate and equilibrium blocks Furthermore, RAC 109 I caused a longer duration of the block than RAC 109 II after the same soaking time and equal periods of washing In other words, for each blocking or washout period, the number of fibres blocked was greater with the former enantiomer Like other local anaesthetics (RITCHIE & RITCHIE 1968, STROBEL & BIANCHI 1970) the compounds were effective in lower concentrations on de-sheathed than on sheathed nerves, this difference probably being accounted for by the removal of the epineural sheath acting as a diffusion barrier

No differences were found between the enantiomers in the rate and extent of accumulation in sheathed nerves at concentrations below and in excess of that required to produce a complete block of nerve conduction The accumulation in the epineural sheath was also the same for the compounds, which indicates a lack of stereo selective binding to connective tissue in the vicinity of the effector cells The uptake of the enantiomers into de-sheathed nerves was identical Thus, the difference in excitation blocks between the

two forms does not seem to be related to stereo selective uptake into the nerve fibre phase However, the access of drug in the biophase is a critical factor It could be that the two forms were distributed differently in the nerve despite the same uptake A possible cause for the higher potency of RAC 109 I would then be less binding to optically active structures en route to the effector cell and vice versa for RAC 109 II However, this seems less credible since the retention of the agents in nerve was identical after varying periods of washing

Alternatively stereo selective binding to adjacent structures did not occur and equal concentrations of the enantiomers were built up in the biophase The findings (ROTH *et al* 1972) that the enantiomers of RAC 109 adsorbed equally to erythrocyte membranes and isolated synaptosomes are interesting in this context The following studies are also illustrative Compounds RAC 109 I and II caused different blocks of electrically induced action potential in single nerve and muscle fibres that possess a minimum of barriers of penetration (ÅKERMAN *et al* 1969, ÅKERMAN & SOKOLL 1969) Previous studies using the voltage clamping technique have contributed to the understanding of the mechanism of action of local anaesthetics (TAYLOR 1959, HILLE 1966 & 1968, NARAHASHI *et al* 1969) As a result of voltage clamp analyses, RAC 109 I was found to reduce the transient change in sodium permeability in single fibres of the sciatic nerve of the frog more effectively than RAC 109 II (HILLE, personal communication) Preliminary findings by NARAHASHI (personal communication) indicate a different potency for the RAC 109 enantiomers regarding the blocking of the action potential in squid giant axon (NARAHASHI *et al* 1970) The findings on single fibres would appear to offer arguments in favour of some stereo selectivity in the mechanism underlying the blocking of nerve conduction

L(+) prilocaine is a slightly more potent local anaesthetic than D(−) prilocaine, but the compounds show the same nerve blocking effect *in vitro* (ÅKERMAN *et al* 1967a) Similar results have been obtained with L(+) and D(−) mepivacaine (ÅBERG & WAHLSTRÖM 1969, ADLER *et al* 1969) The findings on isolated nerve do not support the notion that steric factors are involved at the site of action for local anaesthesia Instead, the quoted studies and those of ÅBERG & ADLER (1970) suggest that some kind of stereo-selective effect on the local vascularity may contribute to the different degree of local anaesthesia obtained with these pairs of enantiomers Thus it would appear as though the differences in local anaesthesia seen in a rather large number of compounds may be related to stereo selectivity in at least two different pharmacological actions of importance for the anaesthetic effect and that the influence of steric factors may vary with the type of compound used

REFERENCES

Aberg, G & B Wahlstrom Vascular effects of mepivacaine *Acta Physiol Scand* 1969, Suppl 330, 71.

Åberg, G & R Adler Thermographic registrations of some vascular effects of a local anaesthetic compound *Svensk Tandläkare Tidskrift* 1970, 63, 671–678

Åkerman B Differing local anaesthetic potency and toxicity of the enantiomers of N n butyl 3 piperidyl 2-chloro-6' methylcarbanilate A new local anaesthetic *Acta pharmacol et toxicol* 1973, 32, 97–111

Åkerman, B, N H Persson & C Tegnér Local anaesthetic properties of the optically active isomers of prilocaine (citanest®) *Acta pharmacol et toxicol* 1967a, 25, 233–241

Åkerman B, G Camougis & R Sandberg Studies with optical antipodes of compounds possessing local anaesthetic activity *Acta pharmacol et toxicol* 1967b, 25, suppl 4 52

Åkerman S B A, G Camougis & R A Sandberg Stereoisomerism and differential activity in excitation block by local anaesthetics *Eur J Pharmacol* 1969, 8 337–347

Åkerman, B & M Sokoll The effect of a pair of enantiomers with local anaesthetic activity on action potential generation and acetylcholine sensitivity in muscle *Eur J Pharmacol* 1969, 8, 331–336

Åstrom, A & N H Persson Some pharmacological properties of *o* methyl-α propyl aminopropionanilide, a new local anaesthetic *Brit J Pharmacol Chemotherap* 1961, 16(1), 32–44

Adler, R, G Adler & G Åberg Effects of optically active isomers and racemate of mepivacaine (carbocaine®) in dental anaesthesia *Svensk Tandlakare Tidskrift* 1969, 62 501–504

Hille, B Common mode of action of three agents that decrease the transient change in sodium permeability in nerves *Nature* 1956 210 1220–1222

Hille, B Pharmacological modifications of the sodium channels of frog nerve *J Gen Physiol* 1968 51, 199–219

Narahashi, T, J W Moore & R N Poston Anesthetic blocking of nerve membrane conductances by internal and external applications *J Neurobiol* 1969 1, 3–22

Narahashi, T D T Frazier & M Yamada The site of action and active form of local anesthetics I Theory and pH experiments with tertiary compounds *J Pharmacol Exp Therap* 1970, 171 32–44

Ritchie, J M & B R Ritchie Local anesthetics Effect of pH on activity *Science* 1968 162, 1394–1395

Roth S, P Seeman, S B A Åkerman & M Chau Wong The action and adsorption of local anesthetic enantiomers on erythrocyte and synaptosome membranes *Biochim et Biophys Acta* 1972, 255, 199–206

Sandberg, R N Aminoalkyl derivatives of spiro succinimides as local anaesthetics *Svensk Kem Tidskr* 1967 79, 577

Schonenberger, H K D Fuchsberger A Petter & R Brinkmann Wechselbeziehungen zwischen Wirkstoff und Rezeptor bei lokalanasthetisch wirksamen N aminoacylephedrinen *Pharm Acta Helv* 1967a, 42, 163–176

Schonenberger, H, A Petter & W Zwez Wechselbeziehungen zwischen Wirkstoff und Rezeptor bei Lokalanasthetica vom Typ der N aminoacylephedrine *Pharm Acta Helv* 1967b 42, 593–603

Shanes, A M & M D Berman Penetration of the intact frog nerve trunk by potassium, sodium chloride and sucrose *J Cell Comp Physiol* 1953 41, 419–450

Strobel, G E & C P Bianchi The effects of pH gradients on the action of procaine and lidocaine in intact and desheathed sciatic nerves *J Pharmacol Exp Therap* 1970 **172**, 1–17

Taylor, R E Effect of procaine on electrical properties of squid axon membrane *Am J Physiol* 1959, **196**, 1071–1078

Telc, A, B Brunfelter & T Gosztonyi Labelled compounds of potential biological interest II – Application of the Yavorsky method for tritiation *J Labelled Compounds* 1971, VIII, 13–23

Truant, A P Local anesthetic studies on isolated frog sciatic nerves *Fed Proc* 1957, **16**, 341

Acta pharmacol et toxicol 1973, 32, 237–245

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Absorption and Elimination of Polychlorinated Biphenyls (PCB) in Goldfish*

By

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Abstract The absorption, accumulation and elimination of PCB (Clophen A50) in goldfish was studied To ensure well defined and reproducible PCB concentrations in the water phase (0 01, 0 05, 0 10 and 0.50 p p m) the experiments were *made in aluminium foil containers* *A rapid uptake and accumulation was observed* but only slight changes in the relative composition of the absorbed PCB s were found In the elimination experiment it was found that the content of PCB in the fish was halved during the first three weeks of the experimental period but that the half life for PCB rose during the experiment

Key words PCB – absorption – elimination – goldfish

Whilst the uptake of PCB by terrestrial fauna occurs mainly via the food, in marine organisms there is the additional possibility of direct absorption through outer surfaces (WILDISH 1970, ZITKO 1970, HANSEN *et al* 1971, HATTULA & KARLOG 1972) Furthermore, fish lack the cytochrome-P-450 enzyme complex (BRODIE & MAICHEL 1962) which is apparently active in the metabolism of chlorinated organic compounds This means that, in contrast to the findings in Japanese quail, pigeon and rat (KOEMAN *et al* 1969, BAILEY & BUNYAN 1972, GRANT *et al* 1971), metabolism of PCB does not take place in fish (HANSEN *et al* 1971)

The subject of the present work is the relationship of the absorption to the water concentration of a highly chlorinated biphenyl mixture (Clophen A 50), and an account is given of attempts which were made to eliminate this mixture in goldfish

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It has proved difficult to achieve well defined and reproducible concen trations of these compounds in the water phase when using glass aquaria, because of PCB's strong adsorption to glass surfaces Special precautions are necessary to avoid this, such as the addition of surfactants (WILDISH 1970, ZITKO 1970), or the use of specially constructed tanks so that the concentra tion in the running water can be controlled (HANSEN *et al* 1971) However, since PCB cannot be adsorbed, or is only slightly adsorbed, to aluminium foil (HATTULA & KARLOG 1971), the experiments on the uptake and elimina tion of PCB in goldfish were made in aluminium foil containers

Material and Methods

Two groups of veil tailed goldfish were used for the experiments The first group used for the absorption experiments consisted of 5 × 16 fish with an average weight of 1 8 g (from 0 70 to 4 60 g) The aquaria were 8 l aluminium foil containers (Ekco 6130) holding 6 l of water (equal parts tap water and de ionized water) with PCB concentra tions of 0 00 0 01 0 05, 0 10 and 0 50 p p m respectively The PCB preparation used was Clophen A 50 (Bayer) dissolved in acetone and containing 1 mg PCB per ml This stock solution was further diluted with acetone so that the calculated amounts of PCB to be added were contained in 3 00 ml acetone An amount of 3 00 ml pure acetone was added for use in the controls

The second group of fish used for the elimination experiment consisted of 90 veil tailed goldfish

All the experiments were carried out at a temperature of 21–23° Sufficient oxygen was ensured by bubbling with pressurised atmospheric air and all the solutions and containers were changed daily Tetra Phyll fish food (PCB content 0 2 p p m) was used as feed and the feeding took place half an hour before the daily changing of the water and the containers in order to reduce as far as possible the presence of particles which could adsorb any of the PCB present Water specimens were taken for analysis as described by *HATTULA & KARLOG (1971) to ensure that the necessary* water concentrations of PCB were obtained Any fish that died spontaneously during the experiments was analysed separately These are not included in the results except where mentioned

Extraction and purification

The following chemicals were used for extraction fat determination and purification of the PCB present Water free Na_2SO_4 Merck (analytical product) Acid washed sea sand Merck n Pentane Uvasol fur Spektroskopie Merck (analytical product) *D ethyl* ether Bie & Berntsen Denmark n Hexane zur Ruckstandanalyse Merck (analytical product) and 95 97 per cent H_2SO_4 Merck (analytical product)

The analysis was made as follows After rinsing with distilled water the whole fish was ground in a porcelain mortar by grinding with water free sodium sulphate and sand (4 0 g sodium sulphate and 5 0 g sand per g fish) after which the powder was dried at room temperature for 48 hours The powder was then transferred to a chromatography column (45 × 2 cm) fitted with a glass filter and washed with a mixture of ether and pentane (1 + 1 and 10 0 ml per g fish) After two hours the solvent was removed and the residue washed with 50 0 ml of ether pentane mixture By this method up to 2 mg

PCB can be extracted quantitatively The extract was evaporated almost to dryness on a water bath (50°) and the residue was transferred to a 10 ml centrifuge tube The rest of the solvent was evaporated and the remainder dried to constant weight in a desiccator The weight of the dried residue was determined and this formed the basis for calculation of the fat content in the analysed samples The residue was then dissolved in hexane (10 mg fat/ml hexane) Equal parts of concentrated sulphuric acid were added to this hexane solution and the mixture was shaken for five minutes Centrifugation was carried out at −15° for 15 minutes (2000 × g) The volume of the clear hexane phase was determined and this solution was used for gas chromatography of the PCB present For the analysis a Varian gas chromatograph Model Aerograf 660, was used This was fitted with EC detector and a 1 5 m long glass column packed with 13/5 QF 1, 4 per cent and 7/5 SF 96 8 per cent on Gaschrome Q 100–120 mesh The content of PCB was determined by calculating the sum of all the peak heights (in mm) and comparing the value with the corresponding sum of Clophen A 50-standard (HOLDEN 1970)

Results

Absorption experiments

Apart from five spontaneous deaths in the groups shortly after the start of the experiment, there were no signs of any toxic effects on the fish in the control group and in the groups with the three lowest concentrations of PCB In the group that was exposed to the highest concentration of PCB (0 5 p p m), the fish lost their appetite after four days in the PCB contaminated water Their stomachs then became swollen, the orange colour became pale, and nine fish died on the seventh day of the experiment Only one fish survived for the whole of the experimental period

Analysis of the fish taken out before the start of the experiment showed that there was background contamination with PCB of 0 2 p p m and contamination with chlorinated pesticides (mainly DDE) of 0 05 p p m Only in the determination of the lowest PCB concentrations found at the beginning of the experiment in groups 1 and 2 was it necessary to use the method described by WESTOO & NOREN (1970) for decomposition of DDE in order to avoid any interference with the PCB determinations

Fig 1 shows the results of the PCB determinations on the fish used in the absorption experiments Each point is the average of the results of analysis of two of the killed fish but not of the spontaneously dead fish It will be seen that the uptake of PCB occurred rapidly, and that as early as after three days, concentrations were observed in all the groups that were more than 100 times greater than those in the water in which the fish were kept At the end of the experiment after 18 days in the contaminated water, the concentrations of PCB in the fish from groups 1–3 were more than 1000 times greater than the corresponding concentrations in the water in which the fish were kept An equilibrium was not yet achieved at that time

Fig 2 shows the calculated content of PCB in the fat tissue of the fish

The values are calculated on the basis of the PCB concentrations shown in fig 1 and the fat determinations made on the individual fish With a fat content of an average of 0 91 per cent (varying from 0 50 to 1 35 per cent) the concentration in the fat tissue of the fish is calculated to be more than 10,000 times greater than the PCB content in the water The highest PCB content found in living fish was 600 p p m (wet weight) or 64 mg PCB per g fat, while the average content in the spontaneously dead fish was 260 p p m (wet weight) or 39 mg PCB per g fat tissue Thus, the concentrations found are in accordance with those found in toxicity examinations in corresponding fish killed by PCB (HATTULA & KARLOG 1972)

Table 1 shows the results of analysis of the water taken during the experiment Three samples were taken from each group at regular intervals during the experimental period The percentage recovery is the average of three analyses and shows that, for the lowest concentrations, a maximum of 10 per cent of the PCB present was adsorbed to the surface of the aluminium containers

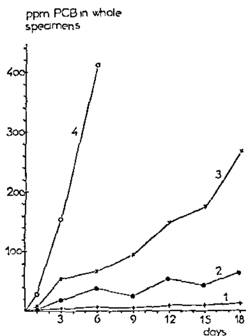


Fig 1 Absorption of PCB by the fish from different concentrations of PCB in water Abscissa Time in days Ordinate Concentration of PCB in whole specimens (p p m)

Water concentration of PCB
1 +——+ 0 01 p p m
2 ●——● 0 05 p p m
3 ×——× 0 1 p p m
4 ○——○ 0 5 p p m

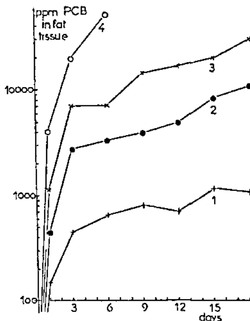


Fig 2 Absorption of PCB by the fish from different concentrations of PCB in water Abscissa Time in days Ordinate Concentration of PCB in fat tissue (p p m in log scale) Water concentration of PCB
1 +——+ 0 01 p p m
2 ●——● 0 05 p p m
3 ×——× 0 1 p p m
4 ○——○ 0 5 p p m

Table 2 shows the total amount of PCB administered to the four groups in the experimental period, and also the amount of PCB absorbed by the killed and spontaneously dead fish in the individual groups, regardless of the fact that the groups did not contain the same number of fish during the whole period

It will be seen that the absorption (both the absolute absorption and the percentage of the amount added) increased the more PCB there was in the

Table 1

Analysis of water samples from containers (figures are average of three series of ex tractions)

p p m PCB in water	0 01	0 05	0 10	0 50
% of recovery	90 2 (89 4–91 0)	96 2 94 5–98 0)	101 7 (100 0–102 5)	110 2 (101 0–115.5)

Table 2

Total absorption of PCB by the fish from water

Concentration of PCB in water (p p m)	0 01	0 05	0 10	0 50
Total amount added (mg)	1 080	5 400	10 800	24 000
Total amount absorbed (mg)	0 148	1 221	3 578	3 592
% of absorption	13 7	22 6	33 1	15 0

water in the three groups, where the number of fish was comparable during the whole period The relatively modest absorption percentage in group 4 can be explained by the deaths that occurred in this group on the seventh day of the experimental period, after which only one fish survived for the whole period

At least eleven well-developed peaks appeared in the gas chromatographic analysis of Clophen A 50, representing at least as many different chlorinated biphenyls In order to ascertain whether any of the compounds found were eliminated or accumulated to any extent in the living organism, five of the highest peaks (Nos 3, 5, 7, 8, 10) were chosen for closer analysis The relationships between the heights of peaks 3 and 5, 5 and 7, and 8 and 10 were calculated both for the 30 chromatograms for the standard solutions (after treatment with concentrated sulphuric acid) and for all the chromatograms taken during the analysis of the fish in the first, second and third weeks of the experiment (64 chromatograms in all) The results of these calculations are shown in table 3, from which it will be seen that no great changes occurred during the experimental period in the relative composition of the chlorinated biphenyls The compound represented by peak 3 seems to be an exception, since the relationship between the heights of peaks 3 and 5 fell during the experimental period, without any corresponding changes being demonstrable in the relationship between peaks 5 and 7

Table 3

Relationships of PCB peaks from living tissue after different periods of incubation compared to PCB standard

Peak no	3 5	5 7	8 10
Standard	1 73	0 98	0 82
Tissue in 1st week	1 53	0 99	0 73
Tissue in 2nd week	1 51	1 06	0 82
Tissue in 3rd week	1 48	0 97	0 82

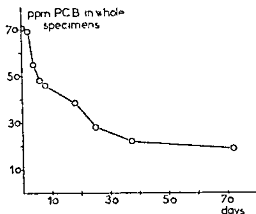


Fig 3 Elimination of PCB from the fish Abscissa Time in days Ordinate Concentration of PCB in whole specimens (p p m)

Elimination experiment

When the fish remained for 13 days in the aluminium foil containers in water containing 0 10 p p m Clophen A 50, a PCB concentration of about 70 p p m accumulated in the fish The fish were transferred to fresh water and after remaining there for three days, the fish were taken out for PCB determinations, to start with, at two-day intervals and later, at longer intervals

The results of the elimination experiment are shown in figs 3 and 4 Fig 3 gives the results of the PCB analyses, each point being the average of the results from five fish This curve shows that the PCB content in the

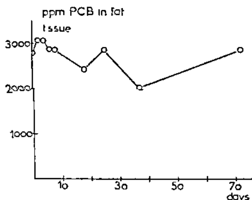


Fig 4 Elimination of PCB from the fish Abscissa Time in days Ordinate Concentration of PCB in fat tissue (p p m)

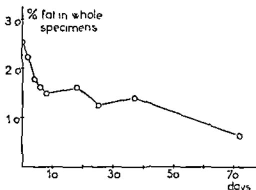


Fig 5 Decrease of fat tissue of the fish during the elimination period Abscissa Time in days Ordinate % fat in whole specimens

fish is halved during the first three weeks of the experiment, but that the half-life for PCB increases during the experimental period A corresponding reduction in the calculated PCB content in the fat tissue of the fish (fig 4) could not be demonstrated, a halving of the PCB concentration not being demonstrable during the ten-week experimental period This disparity can be explained by the decrease found in the fat content of the fish, as shown in fig 5 It will be seen from the figure that a severe reduction took place in the lipoid content during the first few days of the period, so that the calculated PCB content in the fat phase was not decreased in parallel with the PCB content in the whole fish Since the fish showed no signs of loss of appetite during the experimental period, it cannot be excluded that the PCB present may have caused changes in the fat metabolism of the fish, resulting in a decrease in lipoid content

Conclusion and Discussion

The rapid uptake and accumulation of PCB in the goldfish used in this study is in agreement with the results obtained by Hansen *et al* (1971) in experiments with estuarine fish kept in running water containing 0 001–0 005 p p m Aroclor 1254 However, it has not been possible in the present study to obtain a balance between the PCB concentrations in the water and in the fish during the three-week experimental period

The rapid concentration of PCB observed in the fish must raise the question as to whether the daily changing of the water containing PCB is sufficient to maintain a reasonably constant PCB concentration in the experimental period

The slight change found in the relative composition of the absorbed PCB corresponds exactly with the findings of Hansen *et al* (1971) in experiments

with Aroclor 1254 However, it is in contrast to the results of GRANT *et al* (1971) in their experiments on rats, where the relative composition of the Aroclor 1254 used underwent considerable changes with metabolism in the liver Similar metabolism was demonstrated by KOEMAN *et al* (1969) in Japanese quail

If it can be assumed, as is the case with a number of other chlorinated organic compounds, that the cytochrome P-450 enzyme complex is responsible for the metabolism of PCB, the results obtained are in agreement with the observation that this enzyme system is absent in fish but is found in both mammals and birds (BRODIE & MAICKEL 1962)

The results obtained in the elimination experiment are also in agreement with those of HANSEN *et al* (1971), who found that 61 per cent of the PCB taken up was excreted during the course of the 84 days experimental period

REFERENCES

Bailey S & P J Bunyan Interpretation of persistence and effects of polychlorinated biphenyls in birds *Nature* 1972 236, 34–36

Brodie B B & R P Maickel Comparative biochemistry of drug metabolism In *Metabolic factors controlling duration of drug action* Ed B B Brodie & E G Erdos Pergamon Press 1962, pp 299

Grant, D L W E J Phillips & D C Villeneuve Metabolism of polychlorinated biphenyls (aroclor® 1254) mixture in rat *Bull Environ Tox* 1971 6 102–112

Hansen D J P R Parrish J I Lowe, A J Wilson Jr & P D Wilson Chronic toxicity uptake and retention of aroclor ® 1254 in two estuarine fishes *Bull Environ Contam Tox* 1971 6 113–119

Hattula M L & O Karlog Adsorption of polychlorinated biphenyls (PCB) on glass surfaces *Dansk Tidsskr Farm* 1971 45 259–261

Hattula M L & O Karlog Toxicity of polychlorinated biphenyls (PCB) to goldfish *Acta pharmacol et toxicol* 1972, 31 238–240

Holden A V Quantitative estimation of polychlorinated biphenyls (PCB) residues *O E C D Collaborative Study* 1969/70 (1970)

Koeman J H M C ten Noever de Brauw & R D de Vos Chlorinated biphenyls in fish mussels and birds from the River Rhine and the Netherlands coastal area *Nature* 1969 221 1126–1128

Westoo G & K Norén Determination of organochlorine pesticides and polychlorinated biphenyls in animal food *Acta Chem Scand* 1970 24 1639–1644

Wildish D J The toxicity of polychlorinated biphenyls (PCB) in sea water to gammarus oceanicus *Bull Environ Contam Tox* 1970, 5 202–204

Zitko V Polychlorinated biphenyls (PCB) solubilized in water by nonionic surfactants for studies of toxicity to aquatic animals *Bull Environ Contam Tox* 1970 5 279–285

Table 1

Weight of dogs during experiment

Study I Adenine concentration 2.5 %
Rate of infusion 300 or 600 ml/hour (corresponding to 7 5 or 15 g adenine/hour)

Dog no	Sex	Dosage mg/kg b w per day	Days after the start of treatment — Weight (kg) 0	4	11	18	Autopsy		Weight gain or -loss (kg)
3	♂	control	11 3	11 7	11 5	11 8	12 1	day 21	+0 8
10	♀	"	9 1	9 2	9 2	9 5	9 5	" 22	+0 4
11	♀	"	8 1	7 9	7 9	8 1	8 1	" 21	±0
2	♂	10	8 6	8 7	8 7	8 0	8 0	" 18	−0 6
5	♂	10	11 8	11 3	11 3	11 3	11 3	" 18	−0 5
9	♀	10	11 1	10 8	10 5	10 5	10 5	" 18	−0 6
1	♂	35	11 4	11 1	10 9	9 8	9 2	" 22	−2 2
4	♂	35	10 7	10 7	11 0	10 2	10 1	" 21	−0 6
12	♀	35	11 7	10 3	dead		10 4	" 7	−1 3
17	♀	35	13 0	11 3	11 3	dead	10 5	" 14	−2 5
6	♂	85	10 4	9 8	dead		8 8	" 10	−1 6
14	♀	85	11 5	10 2	dead		9.2	" 8	−2 3
16	♀	85	10 3	9 6		8 1			(−2.2)
7	♂	135	9 5	8 7	dead		8 0	" 7	−1 5
8	♂	135	9 7	8 7	dead		7 1	" 11	−2 6
15	♀	135	9 1	8 6	7 1	dead	6 0	" 17	−3 1

Study II Adenine concentration 0 5 %
Rate of infusion 120 ml/hour (corresponding to 0 6 g adenine/hour)

Dog no	Sex	Dosage mg/kg b w per day	Days after the start of treatment — Weight (kg) 0	5	12	Autopsy		Weight gain or -loss (kg)
60	♀	20	11 1	10 3	9 4	9 0	day 19	−2 1
69	♂	20	13 7	13 3	13 0	13 1	" 19	−0 6
93	♂	35	12 4	12 2	11 0	10 7	" 19	−1 7
94	♂	35	10 4	9 4	8 9	8 4	" 19	−2 0

Results

Study I (adenine concentration 2 5 % rate of infusion 300 or 600 ml/hour)

All the dogs behaved normally during the first three days After the fourth day all the dogs on the two higher doses (85 and 135 mg/kg) and one dog on a dose of 35 mg/kg developed a severe reaction They refused food and

Table 2.

Blood urea nitrogen (urastrat chromatography papers – manufactured by Warner Chilcott Laboratories, N J, U S A)

Study I Adenine concentration 2 5 %.
Rate of infusion 300 or 600 ml/hour (corresponding to 7 5 or 15 g adenine/hour)

Dog no	Sex	Dosage mg/kg b w per day	Days after the start of treatment 0	4	7	11	14	18	21
			Urea nitrogen – mg/100 ml blood						
3	♂	control	16	20	18	13	20		18
10	♀	"	19	20	16	18	14	23	18
11	♀	"	18	18	18	13	18	18	19
2	♂	10	16	22	20	20	20		
5	♂	10	15	13	13	15	13		
9	♀	10	24	20	18	23	18		
1	♂	35	13	24	24	23	53	50	65
4	♂	35	15	24	16	18	33	45	48
12	♀	35	21	>75[1]	dead				
17	♀	35	18	55	>75	>75	dead		
6	♂	85	15	>75	>75	dead			
14	♀	85	18	>75	>75	dead			
16+	♀	85	18	>75	>75	>75	>75	60	60
7	♂	135	13	>75	dead				
8	♂	135	19	>75	>75	dead			
15	♀	135	20	>75	>75	>75	>75	dead	

+ surviving dog

Study II Adenine concentration 0 5 %
Rate of infusion 120 ml/hour (corresponding to 0 6 g adenine/hour)

Dog no	Sex	Dosage mg/kg b w per day	Days after the start of treatment 0	5	8	12	15	19
			Urea nitrogen – mg/100 ml blood					
60	♀	20	18	48	35	43	39	49
69	♂	20	15	15	15	18	15	15
93	♂	35	13	25	30	46	46	45
94	♂	35	20	33	34	56	55	>75

[1] It is impossible with this method to determine values exceeding 75 mg/100 ml

water, looked unwell and some of them vomited repeatedly They rapidly lost weight (table 1) and developed diminished skin turgor (dehydration)

Table 3

Serum creatinine (HANRY 1965)

Study I Adenine concentration 2 5 %
Rate of infusion 300 or 600 ml/hour (corresponding to 7.5 or 15 g adenine/hour)

Dog no	Sex	Dosage mg/kg b w per day	Days after the start of treatment 0	4	7	11	14	18	21
			Creatinine – mg/100 ml serum						
3	♂	control	1 1	0 9	1 0	0 9	0 9		0 9
10	♀	"	1 0	0 9	1 1	1 0	1 0	1 1	0 9
11	♀	"	0 8	0 8	0 9	0 8	0 9	1 0	1 0
2	♂	10	0 9	1 0	0 9	0 9	1 0		
5	♂	10	0 9	0 8	0 9	1 0	0 9		
9	♀	10	1 0	1 0	0 8	1 0	0 9		
1	♂	35	0 9	1 7	2.2	2 2	4 2	5 6	8 0
4	♂	35	1 0	1 0	1.2	1 0	1 3	3 3	4 0
12	♀	35	0 9	11 5	dead				
17	♀	35	1 3	4 4	9 2	10 4	dead		
6	♂	85	0 9	11 1	12 3	dead			
14	♀	85	0 9	10 1	11 5	dead			
16+	♀	85	0 8	8 7	9 2	7 8	6 1	4 6	3 6
7	♂	135	0 9	12 8	dead				
8	♂	135	0 9	10 6	12 2	dead			
15	♀	135	0 8	8 3	10 8	9 6	6 4	dead	

+ surviving dog

Study II Adenine concentration 0 5 %
Rate of infusion 120 ml/hour (corresponding to 0 6 g adenine/hour)

Dog no	Sex	Dosage mg/kg b w per day	Days after the start of treatment 0	5	8	12	15	19
			Creatinine – mg/100 ml serum					
60	♀	20	1 0	4 9	4 2	3 5	3 3	4 0
69	♂	20	0 9	0 9	0.9	0 9	0 9	0 9
93	♂	35	0 8	1 7	2 1	2 7	3 0	2.5
94	♂	35	1 1	1 9	3 3	3 8	4 4	4 8

The values of creatinine and urea nitrogen in the blood increased (table 2 and 3) and the specific gravity of the urine remained low indicating an impairment of renal function The adenine infusion was then discontinued but all the dogs except one died or were sacrificed

Table 4

Values for the surviving dog on 85 mg adenine/kg body weight per day (administration was discontinued after four days)

	Days after the start of treatment															
	0	4	7	11	14	18	21	40	46	53	60	69	74	77	81	84*
Weight (kg)	10 3	9 6	–	–	–	8 1	–	8 0	8 1	8 7	8 4	8 3	8 5	–	8 5	8 5
Urine specific gravity	1 020	1 015	1 013	1 013	1 014	1 015	1 018	1 018	1 023	1 022	1 023	1 019	1 027	1 031	1 032	1 016
Serum creatinine (mg/100 ml)	0 8	8 7	9 2	7 8	6 1	4 6	3 6	1 6	1 6	1 5	1 3	1 2	1 2	1 2	1 5	1 3
Blood urea nitrogen (mg/100 ml)	18	> 75	> 75	> 75	> 75	60	60	30	28	25	33	30	33	28	18	34

* autopsy

Table 5

Organ weights in per cent of body weight
Occurance of renal lesions in dogs after administration of adenine

Dog no	Sex	Dosage mg/kg b w per day	Heart	Lungs	Spleen	Liver	Kidneys	Adrenals	Renal lesions
3	♂	control	0 74	0 62	0 40	2 85	0 45	0 011	0
10	♀	"	0 82	0 65	0 92	3 33	0 58	0 012	0
11	○	"	0 98	1 04	0 64	4 63	0 52	0 015	0
2	♂	10	1 16	0 84	0 43	3.58	0 68	0 012	0
5	♂	10	0 88	0 69	0 73	2 31	0 53	0 009	0
9	♀	10	0 72	0 61	0 50	3.27	0 57	0 014	0
1	♂	35	0 91	0 83	–	2 28	0 85	0 015	+
4	♂	35	0 82	0 65	–	3 18	0 80	0 017	+
12	♀	35	1 01	0 75	0 47	3 58	0 77	0 016	+
17	♀	35	1 15	1 81	0 36	4 14	0 72	0 019	+
6	♂	85	1 22	1 44	0 37	4 73	0 89		+
14	♀	85	1 04	1 26	0 92	3 70	0 88	0 014	+
16[1]	♀	85	0 73	0 56	0 43	2 84	0 43	0 018	+
7	♂	135	1 25	0 97	0 25	3 77	0 81	0 016	+
8	♂	135	1 20	1 60	0 24	4 71	0 95	0 023	+
15	♀	135	1 54	2 11*	0 27	3 99	0 90	0 030	+
60	♀	20	0 94	0 73	0 53	2 98	0 46	0 013	+
69	♂	20	0 90	0 64	0 41	2 82	0 57	0 012	0
93	♂	35	1 03	0 82	0 56	3 47	0 80	0 010	+
94	♂	35	1 04	1 32	0 90	3 30	0 63	0 011	+

[1] = Surviving dog
* = The increase in weight was due to an acute purulent bronchopneumonia

The three surviving dogs on 35 mg/kg continued to receive adenine They showed abnormal values for creatinine, urea nitrogen and urine specific gravity The results of all the other tests were within the normal range

One dog on the dose 85 mg/kg survived the crisis and was studied for the reversibility of the symptoms Urine and blood analysis were carried out during the whole experiment Only the values for creatinine, urea nitrogen and specific gravity of the urine were altered and are presented in table 4 When the values had returned to normal levels – 85 days after the start of the treatment – the dog was sacrificed and examined *post mortem*

Study II (adenine concentration 0.5 %, rate of infusion 120 ml/hour)

In this investigation the animals did not react as dramatically as in study I All the dogs lost weight (table 1) but only one of them looked unwell The values for creatinine and urea nitrogen in the blood and the specific gravity of the urine reached abnormal levels in all the dogs but one (on 20 mg/kg table 2 and 3) As in study I all other values were within the normal range

Gross pathology (study I and II)

Varying degrees of dehydration were noted in all the dogs which had died during the treatment period Furthermore hyperaemia and oedema of the gastric mucosa together with an odour of ammonia were noted (uraemic gastritis)

There were macroscopical renal changes in one dog on a dose of 20 mg/kg and in all the dogs on higher doses (table 5) The kidneys were enlarged and pale grey in colour, sometimes of firm consistency They were usually of normal shape but occasionally had a rough surface In most kidneys numerous diminutive yellowish grains were visible in the cortex The surviving dog on a dose of 85 mg/kg (No 16) had kidneys of normal size but the colour was pale the surface granulated and the consistency very firm

The relative weight of the kidneys was increased in all the dogs on 35 mg/kg and upwards (except for the surviving dog on 85 mg/kg) The relative weights of the hearts and adrenals were increased in the dogs on 135 mg/kg (table 5)



Fig 1 Kidney section of a dog given adenine (85 mg/kg body weight per day) for 4 days Crystals of 2 8-dihydroxyadenine can be seen in the renal tubules using polarized light Haematoxylin and eosin (magnification × 110)

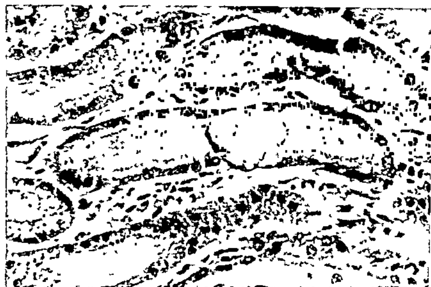


Fig 2 An enlarged view of a crystal as seen in fig 1 (magnification ×440)

Main histopathological findings (study I and II)

Formations of yellowish, rosette-shaped or amorphous birefringent crystals (up to about 40 μ in size) were seen in the uriniferous tubules in one of the two dogs on a dose of 20 mg/kg and in all the dogs on higher doses (fig 1 and 2) The crystals were situated in both the distal and proximal convoluted tubules. A pronounced tubular nephrosis was seen The epithelium was swollen and vacuolated and nuclear changes occurred frequently. Focally in the tubules there was considerable cellular debris due to desquamation of epithelial cells. As a sign of repair some tubules were lined by flat elongated cells The tubules were often slightly to moderately dilated and the glomeruli were in rare cases atrophic As a result of this damage there were focal infiltrations of lymphocytes and polymorphs and an increase in the amount of the interstitial connective tissue. Other pathological changes belonging to the uraemic condition are not specified

The surviving dog on 85 mg/kg was examined almost three months after discontinuing the administration of the adenine. Crystals were still present in the uriniferous tubules At this time fibrosis of the kidneys had developed and the tubular nephrosis remained in small areas.

The dogs on 10 mg/kg did not differ pathologically from the controls.

Discussion

Studies of the metabolic fate of orally and intraperitoneally administered adenine in rats and mice have shown the *in vivo* oxidation of adenine to 2,8-

dihydroxyadenine and deposition of the latter as crystalline occlusions in the renal tubules (BENDICH *et al* 1950, PHILIPS *et al* 1952) In the present investigation crystals of the same microscopic appearence were found in the kidneys of dogs treated with adenine intravenously, indicating that the same metabolic pathways also exist in dogs As the solubility of 2,8 dihydroxyadenine hemihydrate in water is about 2 0 mg per liter (BENDICH *et al* 1950) the crystals were not dissolved by fixation in 10 per cent neutral formalin, even after several months nor were they affected by other dissolving agents used in histological technique There is, however, a possibility that some of the crystals are lost during sectioning, so that the actual number of crystals would be higher than that observed

All the dogs with crystalline deposits developed impaired renal function as reflected by an increase in blood urea nitrogen and creatinine This could have been due to changes in renal blood flow, changes in glomerular filtration rate, metabolic effects on the tubular cells causing changes in their reabsorbtion or secretion, mechanical damage to the tubular epithelium by crystals or occlusion of the tubular lumina by crystals and cell debris In the present study, mechanical damage to the tubular epithelium and occlusion of the tubular lumina seem to be the main causes of the impairment in renal function Crystals of 2,8-dihydroxyadenine were formed in the distal and later in the proximal tubules as a consequence of saturation when water is reabsorbed from the tubules The occlusion of the tubular lumina leads to an increased intratubular hydrostatic pressure followed by a diminished glomerular filtration rate and a retention of blood urea nitrogen The microscopically observed changes in the renal tissue are primarily a degeneration of the tubular cells and a dilatation of the tubules At a later stage there is infiltration by inflammatory cells and an increase in interstitial connective tissue

In the surviving dog on 85 mg/kg body weight the values for blood creatinine and urea nitrogen decreased and there was a minor weight recovery The histopathological renal changes, however, were of a chronic type (fibrosis) indicating irreversibility of the changes

In this investigation the "highest safe dose" after repeated intravenous administration to dogs was considered to be 10 mg adenine/kg body weight per day A reduced rate of infusion and a lower concentration of adenine did not change its effect on the kidneys

This "safe" dose in dogs corresponds closely to that in human patients receiving ACD adenine blood In the majority of instances the number of units given will rarely exceed 30 which roughly corresponds to a dose of 15 mg adenine/kg body weight This dose has not been proved to have any harmful effects (FALK, LINDBLAD & WESTMAN, unpublished results)

REFERENCES

Bendich, A G B Brown, F S Philips & J B Thiersch The direct oxidation of adenine *in vivo* *J Biol Chem* 1950, **183**, 267–277

de Verdier, C. H, L Garby, M Hjelm, C Hogman & A Eriksson Adenine in blood preservation Post transfusion in viability and biochemical changes *Transfusion* 1964a, **4**, 331–338

de Verdier, C H, C Hogman, L Garby & J Killander Storage of human red blood cells II The effect of pH and of the addition of adenine *Acta Physiol Scand* 1964b, **60**, 141–149

Hanry, H Untersuchungen zur Bestimmung von Kreatinin im Serum *Ärztl Lab* 1965, **11**, 175–180

Philips, F S, J B Thiersch & A Bendich Adenine intoxications in relation to *in vivo* formation and deposition of 2,8 dioxyadenine in renal tubules *J Pharmacol Exp Therap* 1952, **104**, 20–30

Shields, C E, H Lopas & N I Birndorf Investigation of nephrotoxic effects of adenine and its metabolic product, 2 8 dioxyadenine, on primates (Macaca Irus) *J Clin Pharmacol New Drugs* 1970, **10** 316–322

Acta pharmacol et toxicol 1973, 32, 257–265

From the National Veterinary Institute, Oslo Norway

Methaemoglobin Formation by Diamino Metabolites of DNOC and DNBP

By

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Abstract The diaminophenols DAOC and DABP have been investigated for their methaemoglobin forming effect. Both substances were found to be active methaemoglobin inducers *in vitro* as well as *in vivo* on goats and pigs After intraperitoneal administration of 33 mg/kg (DAOC) and 43 mg/kg (DABP) of the diaminophenols the animals died within one hour Administered in this way DAOC produced a lethal methaemoglobinemia within 15–20 minutes while after intraruminal administration the methaemoglobinemia developed less dramatically At corresponding doses DABP produced almost the same level of methaemoglobinemia in the animals In a more prolonged experiment haemolysis and acute nephrosis were observed and haemoconcentration occurred in all the animals It is concluded that the diaminophenol metabolites are responsible for the methaemoglobin formation following dinitrophenol poisonings in ruminants

Key words Methaemoglobinemia – haemolysis – dinitrophenols metabolism – goats – swine

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The dinitrophenol DNOC (2-methyl-4,6 dinitrophenol), and especially DNBP (2-(1-methyl n-propyl)-4,6-dinitrophenol), induce methaemoglobinemia when given intra-ruminally to cattle (FROSLIE & KARLOG 1970) Dinobuton, an isopropyl carbonate ester of DNBP, also produces methaemoglobinemia when given by the intra ruminal route (FROSLIE 1971c)

Although HENNEBERG (1971) demonstrated moderate methaemoglobinemia in rats experimentally poisoned with DNBP and DNPP (2 isopropyl 4,6 dinitrophenol), this methaemoglobinemia following 4,6-dinitrophenol poisoning seems to be of significance for the ruminants only FROSLIE & KARLOG (1970) related this phenomenon to the reduction process which occurs in the rumen, since the aminophenols formed belong to a group of chemicals including a number of methaemoglobin forming compounds (KIESE 1966)

In agreement with this it was shown that the 6-amino metabolites of both DNOC and DNBP had a potent methaemoglobin-forming effect *in vitro* (FRÖSLIE 1971a). However, the results of the investigations performed on sheep and swine excluded these metabolites as inducers of the methaemoglobinemia by dinitrophenol poisoning in the ruminants (FRÖSLIE 1971b) It was then thought that the diaminophenol metabolites, the main end products from the ruminal metabolism of the dinitrophenols (FROSLIE & KARLOG 1970, JEGATHEESWARAN & HARVEY 1970), might be the actual methaemoglobin-forming compounds

In this paper a description of the results of the experiments with the diaminophenols in goats and pigs is presented

Materials and Methods

Reference substances

2-methyl-4,6-diaminophenol (4,6–diamino-o-cresol, DAOC)

2-(1 methyl n propyl)-4,6 diaminophenol (4,6 diamino-2 sec -butylphenol, DABP)

These substances were made from the corresponding dinitrophenols by reduction with hydrogen and 5 per cent palladium catalyst on charcoal For isolating the hydrochloride salts of the diamines, the dinitrophenols (1–2 g), the catalyst (about 1 g), and 50 ml of ethyl acetate were shaken under hydrogen at room temperature and atmospheric pressure The calculated volume of hydrogen (1.35 l per 10^{-2} mol of dinitrophenol) was absorbed within one hour, most of it during the first 20 minutes The catalyst was removed by filtration on celite under nitrogen atmosphere Dry hydrochloride gas was then bubbled through the solution The dihydrochloride salt of DAOC was precipitated as a white powder and could be isolated, but the salt of DABP could not be isolated in this way Therefore, for the *in vivo* experiments especially, the reduction was carried out in a solution of absolute ethanol, and the reaction mixture including the catalyst was injected directly into the animals The solutions turned dark red immediately if oxygen was present, so the injections were made under reducing atmosphere

Experimental methods

The *in vitro* experiments on bovine erythrocytes were carried out as described previously (FRØSLIE 1971a) The solutions of aminophenols were kept under a nitrogen atmosphere until incubation Since DABP could not be isolated in a stable form, the experiments with this compound were carried out as pilot experiments only

The *in vivo* experiments were carried out on 4 goats of Norwegian origin (22–30 kg b w) and 2 two-months-old pigs of Norwegian Landrace origin (22,24 kg b w) The test solutions were injected directly into the rumen or intraperitoneally by a needle Blood samples were taken at given intervals by venipuncture

Analytical methods

The haemoglobin concentrations were determined by the cyanmethaemoglobin method, the concentrations of methaemoglobin as described in STEWART & STOLMAN (1961), and haematocrit by a micro method ("Cellokrit 2" centrifuge, AB Lars Ljungberg & Co, Stockholm)

Results

Effect of the diaminophenols on bovine erythrocytes in vitro

The results of the incubations of bovine erythrocytes with DAOC at concentrations of 5×10^{-4}M are presented in fig 1 As shown in the figure, DAOC had a clear methaemoglobin forming effect on the erythrocytes At the concentrations used, more than 50 per cent of the haemoglobin (of a total of about 3×10^{-3} M) was oxidized within 15 minutes

DABP also showed a methaemoglobin forming effect, but the instability of this substance made it difficult to carry out more exact experiments

Effect of DAOC on goats

The results of these experiments are presented in fig 2 and in table 1

The dose of DAOC used in these experiments (33 mg free amine/kg) killed both the goats When given by an intraperitoneal injection, DAOC almost immediately gave a marked methaemoglobinemia in the goat, with a lethal level of methaemoglobin (about 80 per cent of total Hb) as early as 15 minutes after administration As the methaemoglobinemia developed, the goat fell down with dyspnoea and coma and died after 45 minutes As shown in the table there was a clear increasing tendency in the haemoglobin and

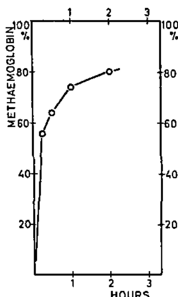


Fig 1 Methaemoglobin formation *in vitro* in bovine erythrocytes after incubation with DAOC at concentrations of 5×10^{-4} M The curve indicates the mean values of 4 series of experiments

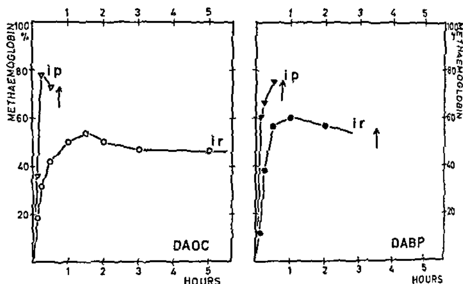


Fig 2 Methaemoglobin concentrations in the blood of goats after intraperitoneal (ip) and intra ruminal (ir) application of DAOC and DABP Doses DAOC 33 mg/kg DABP 43 mg/kg (free amine) The arrows indicate mors

haematocrit values during the experiment By *post mortem* examination no gross lesions except the dark brown blood and the haemoconcentration were found

By ruminal administration the same dose of DAOC gave a somewhat slower development of the methaemoglobinemia, with a maximum level of methaemoglobin of 54 per cent after one hour and a half, and death occurred after 16–20 hours The methaemoglobinemia remained at about the same level for 4–5 hours, and an increasing dyspnoea and cyanosis developed. Three hours after drug administration haemolysis was observed in the blood samples, and in the table it can be seen that a serious haemoconcentration gradually developed In the course of 4 hours the level of haemoglobin and packed red cell volume increased to about twice their initial value of 12 0 g/100 ml and 37 per cent, respectively At *post mortem* examination methaemoglobinemia and haemoconcentration were found, and there were acute nephrotic lesions in the kidneys Similar nephrotic lesions of varying degrees were observed earlier after administration of the dinitrophenols in question or their 6 amino metabolites intra-ruminally to sheep (own observations)

Effect of DABP on goats

The results of the two experiments are shown in fig 2 and table 1

The effect of DABP on the goats was very similar to that of DAOC A

Table 1

Haemoglobin (g/100 ml blood, Hb) and haematocrit (vol % packed red cells, PCV) values in goats and pigs after intraperitoneal (i p) or intra ruminal (i r) administration of DAOC and DABP Doses DAOC 33 mg/kg DABP 43 mg/kg (free amine)

	DAOC						DABP					
Hours	Goat i p		Goat i r		Pig i p		Goat i p		Goat i r		Pig i p	
	Hb	PCV	Hb	PCV	Hb	PCV	Hb	PCV	Hb	PCV	Hb	PCV
0	12 4	38	12 0	37	10 5	35	12 4	38	12 5	37	11 1	36
⅛	12 8	38	12 0	38	11 1	36	13 0	39	12 8	40	12 4	37
¼	17 0	50	12 0	38	13 9	40	15 2	49	14 4	43	14 4	43
½	—	—	12 0	38	16 5	48	19 6	65	14 8	45	18 4	56
1			13 6	45	—	—	—	—	15 2	45	—	—
2			17 5	56					22 5	67		
3			21 0	61					—	—		
5			22 2	68								

dose of DABP of 43 mg/kg (a corresponding molar dose to DAOC) also resulted in a lethal level of methaemoglobinemia within only 15–20 minutes after the intraperitoneal administration The goat died after 45 minutes in severe dyspnoea and cyanosis, and as shown in the table a considerable degree of haemoconcentration developed during this time

Intra-ruminal administration of the same dose had a somewhat weaker effect, with a maximum level of methaemoglobin of 60 per cent after one hour The level of total haemoglobin and haematocrit gradually increased to about twice their initial values With increasing signs of dyspnoea and cyanosis, the goat died after 3 hours and a half

In neither of these goats was haemolysis observed in the short time after the administration of DABP, and at *post mortem* examination no gross lesions except the methaemoglobinemia and the haemoconcentration were observed

Effect of DAOC and DABP on pigs

The results of these experiments are presented in fig 3 and table 1

Both DAOC and DABP at the above mentioned doses killed the pigs with methaemoglobinemia within one hour after the intraperitoneal administration As shown in the figure the methaemoglobinemia developed very rapidly, and lethal levels of methaemoglobin were reached within 15–20 minutes as in the goats As shown in the table, a considerable haemoconcentration also occurred in the pigs during the short time of the experiments At *post mortem* examinations only methaemoglobinemia and haemoconcentration were observed

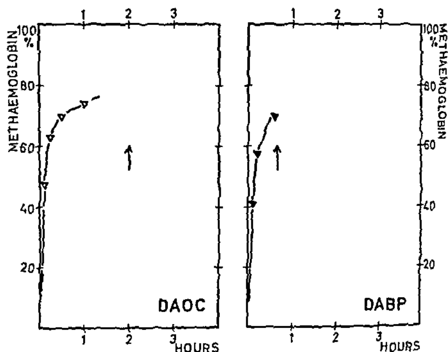


Fig 3 Methaemoglobin concentrations in the blood of pigs after intraperitoneal administration of DAOC and DABP Doses DAOC 33 mg/kg, DABP 43 mg/kg (free amine) The arrows indicate mors

Discussion

The 4,6 dinitrophenols DNOC and DNBP have a special effect including methaemoglobinemia and haemolysis when given intra-ruminally to ruminants, while the hyperthermia, typical for these poisonings in monogastric animals and man, is not observed (FRØSLIE & KARLOG 1970) This special effect of the dinitrophenols is connected with their degradation in the rumen to amino metabolites

The reduction process, however, proceeds in two steps, and it was appropriate to find out which of these reduction products i e the mono- or the diaminophenols was the active one Both these compounds belong to groups known for their activity against haemoglobin (KIESE 1966)

The diaminophenol metabolites are not detected in the blood, but since they are found to be the main end product from the ruminal metabolism of the dinitrophenols, they could probably be the active factors JEGATHEESWARAN & HARVEY (1970) found 5 times more conjugated DAOC than 6-ANOC (2-methyl-4-nitro-6-aminophenol) in the urine of sheep after intraruminal administration of DNOC

The monoamine metabolites, however, are detected in the plasma after intra-ruminal administration of DNOC and DNBP, 6-ANOC in a N-acetylated form (FRØSLIE & KARLOG 1970, FRØSLIE 1971b), and both 6-ANOC and 6-ANBP (2 (1-methyl-n propyl)-4-nitro-6-aminophenol) have a potent methaemoglobin-forming effect *in vitro* (FRØSLIE 1971a) From this it is reasonable to assume that the monoamines induce at least partly the methaemoglobinemia following dinitrophenol poisoning. However, investigations on sheep and swine showed that this is not the case (FRØSLIE 1971b) The monoamines must be further reduced in the rumen to produce their activity Their lack of effect *in vivo*, in spite of their effect *in vitro* could be explained by their marked binding to the plasma proteins and their elimination rate from the blood The acetylation of 6-ANOC could also explain its detoxication in the animals

The methaemoglobinemia following dinitrophenol poisoning in ruminants must thus be due to the diaminophenol metabolites, and the experiments presented here confirm this hypothesis Doses of the diaminophenols of 33 mg/kg respective 43 mg/kg (free amine) given intraperitoneally, which clearly exceed the minimum lethal doses, killed all the animals within one hour, while corresponding molar doses of 6 ANOC and 6-ANBP had no clinical effect at all (FRØSLIE 1971b)

The reduction of the dinitrophenols to monoamines, as first pointed out by HARVEY (1958), is accepted as a "protective action of rumen organisms" (CLARKE & CLARKE 1967), but this is actually not the case for the last reduction step On the contrary the reduction of the monoamines to diamines increase the toxicity by a great margin, and is a good example of a metabolic activation of a poison Further experiments are necessary to determine the exact toxicity of the diaminophenols, but the experiments performed indicate that they have almost the same toxicity as the dinitrophenols themselves

The diaminophenols were active both when given intra-ruminally and intraperitoneally, and a somewhat more rapidly occurring effect was observed following the last mentioned mode of administration By using this mode of administration, the methaemoglobinemia set in almost immediately This was also the case for many aminophenols, a result which is of interest for the therapeutic use of methaemoglobinemia formation (KIESE & WEGER 1969) The aminophenols 6 ANOC and 6-ANBP on the other hand, induced methaemoglobinemia only when given intra-ruminally, and after about one hour, in agreement with the finding that they must first be reduced to the diaminophenols All these facts indicate that it is the diaminophenols which are the actual methaemoglobin forming compounds

OGINO & YASUKURA (1957) investigated the metabolism of 2,4-dinitrophenol in order to isolate the cataractogenic metabolite (s) in guinea pigs They found that smaller quantities of the dinitrophenol were excreted as

2 4 diaminophenol which was partly acetylated to 2 amino 4 acetamidophenol and partly oxidized to 2 amino p quinoneimine The diaminophenols DAOC and DABP may be oxidized in a similar way too and since quinones are known for their methaemoglobin forming effect (KIESE 1966) such an oxidation could perhaps influence the formation of methaemoglobin by the poisonings in question

The sec. butylphenols DNBP and 6 ANBP as shown in earlier experiments have when given intra ruminally a distinct higher toxicity than the corresponding orthocresols In spite of the fact that the diaminophenols were given in too large doses to determine their relative toxicity the investigations do not indicate such a great difference between the diaminophenols Therefore the difference in the methaemoglobin forming effect must be due to the production or the metabolism of these diamino metabolites rather than the reactivity of the phenols themselves There is no significant difference in the production of the diaminophenols (FROSLIE & KARLOG 1970) and probably not in their absorption from the rumen The metabolism of the diaminophenols however has not been sufficiently investigated As mentioned above JEGATHEESWARAN & HARVEY (1970) determined conjugated DAOC in the urine of sheep after ruminal dosing of DNOC but their technique (acid hydrolysis) could not determine what kind of conjugation or in what position The earlier observed difference with regard to the N acetylation in the 6 position of the monoamines 6 ANOC and 6 ANBP is of interest in this connection as a similar difference in the metabolism of the diamines could possibly explain some difference in their activity

The most important clinical effects of the diamino metabolites of DNOC and DNBP and of the dinitrophenols themselves and their 6 amino metabolites if given intraruminally are undoubtedly the formation of methaemoglobin the haemolysis and the haemoconcentration occuring in somewhat varying degrees depending on the doses and substances given Nephrotic lesions are also observed but the pathology of these poisonings on the whole must be further investigated

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REFERENCES

Clarke E. G C. & M L Clarke *Garner's veterinary toxicology* 3rd ed Baillere London 1967 p 220

Frøslie A · Methaemoglobin formation *in vitro* by 6-amino metabolites of DNOC and DNBP *Acta pharmacol et toxicol* 1971a, 29, 490–498
Frøslie, A The difference in methaemoglobin formation by 6 amino metabolites of DNOC and DNBP in ruminant and nonruminant animals with special reference to sheep and swine *Acta pharmacol et toxicol* 1971b, 30, 238–247
Frøslie, A Ruminal metabolism and methemoglobin forming effect of dinobuton in sheep *Acta vet scand* 1971c, 12 300–302
Frøslie, A & O Karlog Ruminal metabolism of DNOC and DNBP *Acta vet scand* 1970, 11, 114–132
Harvey, D G Some aspects of the metabolism of 4,6-dinitro *o*-cresol (DNC) by the ruminant *J comp Path* 1958, 68, 54–63
Henneberg M Méthémoglobinémie, coproporphyrinurie et urobilinogénurie chez le rat au cours de l'intoxication expérimentale par les herbicides dérivés du dinitrophenol DNBP (Dinosèbe) et DNPP (Dinoprope) *Europ J Toxicol* 1971, 4, 9–18
Jegatheeswaran, T & D G Harvey The metabolism of DNOC in sheep *Vet Rec* 1970, 87, 19–20
Kiese, M The biochemical production of ferrihemoglobin forming derivatives from aromatic amines and mechanisms of ferrihemoglobin formation *Pharmacol Rev* 1966, 18, 1091–1161
Kiese, M & N Weger Formation of ferrihaemoglobin with aminophenols in the human for the treatment of cyanide poisoning *Europ J Pharmacol* 1969, 7, 97–105
Ogino, S & K Yasukura Biochemical studies on cataract VI Production of cataracts in guinea pigs with dinitrophenol *Amer J Ophthal Ser 3* 1957, 43, 936–946
Stewart, C P. & A. Stolman *Toxicology Mechanisms and analytical methods* Vol II Academic Press, London 1961, p 788–789

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The Irritant Potential of Dilute Solutions of *ortho*-Chlorobenzylidene Malononitrile (CS) on the Eye and Tongue

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Abstract Median threshold concentrations for the riot control agent *o* chlorobenzylidene malononitrile (CS) to produce blepharospasm were found to be $5\ 9\times10^{-6}$M for the rabbit, $2\ 2\times10^{-5}$M for the guinea pig and $3\ 2\times10^{-6}$M for man The threshold concentration to produce sensation on the human cornea was $7\ 3\times10^{-7}$M A dose response relationship was demonstrated Extrapolation of log probit plots for sensation on the human eye suggest that $2\ 7\times10^{-5}$M would be incapacitating The median threshold concentration of CS required to produce sensation on the human tongue was $6\ 8\times10^{-6}$M, a concentration 9 4 times that required to produce eye sensations A dose response relationship on the tongue was demonstrated The use of irritant tests species variations in response, and the effects of solvents on the response are discussed

Key words *Ortho* Chlorobenzylidene – local irritation – eye – tongue

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o Chlorobenzylidene malononitrile (CS), the structural formula of which is shown in fig 1, is the active irritant material in a variety of devices used for the control of riots It is about 10 times more potent an irritant than the previously used harassing agent chloroacetophenone (CN, 'tear gas'), but is significantly less toxic (Himsworth Report 1971) The majority of devices used to disperse CS take the form of cartridges or grenades in which CS is mixed with a pyrotechnic composition which, on ignition, burns and expels CS in the form of a vapour that condenses into minute liquid droplets or particles The clouds of CS so produced are of a low order of inhalation toxicity (Ballantyne & Callaway 1972), but nevertheless concentrations as small as 4 mg/m³ of air make even determined subjects move away from a contaminated area because of lachrymation, salivation, rhinorrhoea, blepharospasm, burning sensations in the eye, mouth, nose, throat and lungs, as well as constricting sensations in the chest (Himsworth Report 1971)

Since CS dose not have a characteristic smell or true taste, it has been

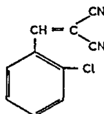


Fig 1 Structural formula of o-chlorobenzylidene malononitrile (CS)

suggested that it may act as a potent non specific stimulant of the common chemical sense (KEELE 1962, CURRIE *et al* 1963) Threshold concentration studies for irritant response to aerosols, and comparison of the relative potencies of different irritants dispersed as aerosols or gases may be undertaken (PUNTE *et al* 1962, OWENS & PUNTE 1963, ALARIE 1966) Such tests, however, are time consuming and require a suitable large exposure chamber with appropriate facilities for sequentially sampling the airborne concentration of the irritant Such exposures may be complicated by the simultaneous appearance of several signs and symptoms, the development of tolerance, and sampling errors In order to screen the relative potencies of several irritant materials, tests are required that are easy to perform, reproducible, and preferably limited to responses in one tissue As a necessary preliminary to studying the mode of action of irritant substances, and comparing their relative potencies, we have measured the threshold concentrations of CS in solution required to elicit irritant sensations on the eye and tongue of man In addition the threshold concentrations of CS required to produce blepharospasm in the rabbit, guinea pig and man have been determined

Materials and Methods

Threshold concentrations of CS in solution necessary to produce irritant effects were estimated as the EC50 (effective concentration 50) The EC50 is the concentration of CS which is required to produce an irritant response in 50 % of individuals of a given species In the rabbit and guinea pig the EC50 was determined for blepharospasm and in man the EC50 was determined for blepharospasm and elicited sensations on the eye and tongue

EC50 estimates on the eye

For the tests on the eyes of both animals and man CS was dissolved in normal saline Because of the relatively rapid hydrolysis of CS a solution of CS was prepared in ethanol and diluted with saline immediately before being applied to the eye Re crystallized CS was weighed out into dry tubes and then dissolved in pure dry ethanol to give concentrated solutions These concentrated solutions were appropriately diluted with dry ethanol to give a test range of concentrations a thousand times that to be

applied to the eye Immediately before use, the alcoholic solutions were diluted a thousandfold with normal saline, so that the final concentration of ethanol in solution was 0 1 % Comparison of the blepharospasm inducing properties of a variety of compounds dissolved in ethanol containing and ethanol free solutions have demonstrated that solutions containing up to 2 % ethanol have no effect on the EC50 for blepharospasm (SWANSTON & WARDROP, unpublished results) The range of concentrations of CS applied to the eye were as follows

Man $2\,5 \times 10^{-7}$ to $8\,0 \times 10^{-6}$ M, rabbit $6\,25 \times 10^{-6}$ to $2\,0 \times 10^{-4}$ M, and guinea pig $1\,77 \times 10^{-5}$ to $2\,98 \times 10^{-5}$ M

Tests for blepharospasm were carried out on 10 rabbits and 10 guinea pigs by a sequential procedure This involved dropping solutions of increasing concentration onto the surface of the cornea until an effect was just observed, the volume of each droplet applied was 0 01 ml After the initial blink reflex, the eye was observed for a period of 10 seconds for the guinea pig and 30 seconds for the rabbit The responses were recorded as follows

Full response – a continuous blepharospasm, or an intermittent blepharospasm consisting of at least 3 tight blinks

Half response – sustained incomplete closure of the eyelids, or at least 2 blinks which excluded the initial reflex blink

The human studies were carried out on young adult male volunteers who were free from allergies and did not have a history of injury or disease of the eye Each subject was given a full medical examination before the tests A total of 10 men were used, and the procedure was similar to that used with the experimental animals Following the application of the 0 01 ml droplets to the surface of the cornea the subjects were asked to describe both the quality and intensity of any sensation they experienced. The time to onset and duration of any sensation elicited was noted Sensation was recorded according to the following scale 0 – no effect, 1 – transient slight effect, 2 – definite sensation, and 3 – marked prolonged sensation with discomfort

Blepharospasm was recorded on the following scale 0 – no effect, 1 – brief intermittent closure of the lids, and 2 – sustained tight closure of the lids

In order to obtain the concentration response data for calculation of the EC50 for both sensation and blepharospasm a Grade 1 effect was counted as a half response and the higher grades were recorded as whole responses The cumulative responses for a given concentration were rounded off to the nearest whole number The EC50, with 95 % confidence limits was calculated by probit analysis (FINNEY 1962)

EC50 estimates on the tongue

The EC50 for sensation on the tongue was determined using 20 men As in the tests on the eye a series of ethanolic solutions of varying concentrations were prepared, and diluted a thousandfold with saline immediately before being applied to the tongue in a sequential procedure Droplets, 0 01 ml in volume, were applied to the tip of the tongue with the mouth kept open The concentration range applied was between 1×10^{-6} M and $1\,6 \times 10^{-5}$ M By using a colour card system, the open mouthed subjects were asked to indicate the onset, duration and degree of discomfort Sensation was recorded according to the following scale 0 – no effect, 1 – slight tingling sensation (recorded as a half response), and 2 – definite strong or burning sensation (recorded as a full response)

As with the observation on the eye, cumulative scores were rounded down to the nearest whole number, and the EC50 calculated by probit analysis

In an attempt to define a relationship between the concentration of CS in solution

Table 1

Threshold irritant response of the eyes of the guinea pig rabbit and man to solutions of CS in saline

	Blepharospasm						Sensation	
	Guinea pig		Rabbit		Man		Man	
	Concentration of CS (molar)	Response rate (1)	Concentration of CS (molar)	Response rate (1)	Concentration of CS (molar)	Response rate (1)	Concentration of CS (molar)	Response rate (1)
	1.77×10^{-5}	2/10	6.25×10^{-6}	0/10	2.5×10^{-7}	0/10	2.5×10^{-7}	2/10
	2.10×10^{-5}	4/10	1.25×10^{-5}	1/10	5.0×10^{-7}	0/10	5.0×10^{-7}	3/10
	2.50×10^{-5}	8/10	2.50×10^{-5}	2/10	1.0×10^{-6}	2/10	1.0×10^{-6}	6/10
	2.98×10^{-5}	9/10	5.00×10^{-5}	5/10	2.0×10^{-6}	4/10	2.0×10^{-6}	9/10
			1.00×10^{-4}	6/10	4.0×10^{-6}	5/10	4.0×10^{-6}	9/10
			2.00×10^{-4}	9/10	8.0×10^{-6}	8/10	8.0×10^{-6}	10/10
EC50 with 95 % confidence limits	$2.2\ (1.9-2.4)\times10^{-5}$		$5.9\ (3.8-10.0)\times10^{-5}$		$3.2\ (2.1-6.1)\times10^{-6}$		$7.3\ (4.2-11.2)\times10^{-7}$	

(1) Response rate recorded as $\frac{\text{number of positive responses}}{\text{Number of eyes tested}}$

and both the onset and duration of sensation elicited solutions of varying concentrations above the threshold value were used Twelve men each had applied to their tongues 0 01 ml drops of 0 0001 %, 0 001 %, 0 01 % and 0 1 % CS dissolved in equal parts by volume of polyethylene glycol 300 and water This solvent system was chosen because of the low solubility of CS in pure water, and because polyethylene glycol 300 is pharmacologically inert in the volumes used All the solutions were applied to the anterior portion of the dorsum of the tongue within one minute of preparation Using a colour card system, the following 4 point scale was used for recording the nature of the sensations produced by these suprathreshold concentrations of CS 0 – no effect 1 – slight stinging or tingling sensation, 2 – mild burning sensation and 3 – strong burning sensation

Results

A summary of the threshold irritant response of the eyes of the guinea pig, rabbit and man to solutions of CS in saline is given in table 1 The EC50 for blepharospasm in the guinea pig was readily estimated at $2\,2 \times 10^{-5}$ M With rabbits, using a 30 second observation period, it was noted that the closure of the eyelid was slower and more deliberate, and occured at intervals of several seconds Nevertheless, a satisfactory dose-response relationship was obtained (table 1), giving an EC50 of $5\,9 \times 10^{-5}$ M

Following the application of irritant solutions of CS to the surface of the cornea, all the men described the sensation as stinging at the lower effective concentrations and burning at the higher concentrations The time to onset of these sensations for individual men varied between 1 and 10 seconds Although there appeared to be a general tendency for the mean time to onset of sensation to decrease with increasing concentration, this was not significant for the lower consecutive concentrations However, the time to

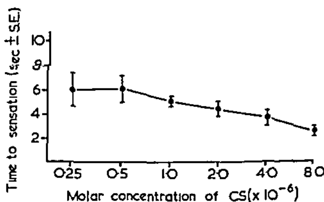


Fig 2 Effects of concentration of CS in saline on the time to onset of sensation following the application of 0 01 ml droplets to the surface of the human cornea

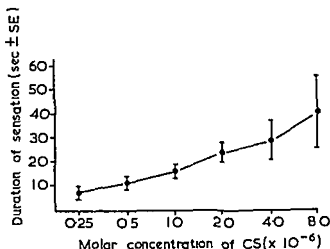


Fig 3 Effect of concentration of CS in saline on the duration of sensation produced after 0 01 ml of solution was applied to the surface of the human cornea

onset of sensation with the higher concentrations was significantly shorter than with the lowest concentrations (fig 2) There was a more definite relationship between concentrations of CS and the duration of sensation, the *duration increasing with higher concentration (fig 3) The relationship* between percentage response and concentration for each grade of effect

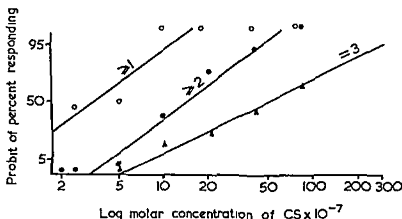


Fig 4 Log probit plot showing the relationship between percentage response and concentration of CS applied to the human eye for each grade of sensation ○ = greater than or equal to grade 1 (transient slight sensation) ● = greater than or equal to grade 2 (definite short lived sensation) △ = grade 3 (marked prolonged sensation with discomfort)

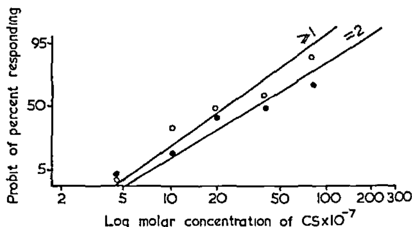


Fig 5 Log probit plot showing relationship between percentage response and concentration of CS applied to the eye for both grades of blepharospasm ○=greater than or equal to grade 1 (intermittent closure of the lids) ●=grade 2 (sustained tight closure of the lids)

recorded is shown as a log probit plot for sensation in fig 4 and for blepharospasm in fig 5

The threshold irritant response of the tongue to solutions of CS in saline is given in table 2, from which the EC50 for sensation was calculated as $6\ 8\times10^{-6}$ M The subjects described the sensation as being initially stinging and then changing to a burning sensation within a few seconds Over the concentration range of CS used to measure threshold values, there was a wide variation in both the time to onset and total duration of sensation elicited

Table 2

Threshold irritant response of the human tongue to solutions of CS in saline

Concentration of CS (molar)	Response rate (1)
1×10^{-6}	0/20
2×10^{-6}	3/20
4×10^{-6}	6/20
8×10^{-6}	11/20
16×10^{-6}	8/10

EC50 (with 95 % confidence limits) = $6\ 8\times10^{-6}$ ($5\ 0-10\ 6\times10^{-6}$)

(1) Response rate recorded as $\frac{\text{number of positive responses}}{\text{number of tongues tested}}$

Concentration of CS (%)

Concentration of CS (%)
0·0001
0·001
0·01
0·1

Mean time to onset of sensation (sec)
Mean duration of sensation (sec)

40 20 0 20 40 60 80 100 120
Onset of sensation (sec) Duration of sensation (sec)

Fig 6 Mean time to onset and mean duration of sensation on the human tongue following the application of 0 01 ml of various cnocentrations of CS dissolved in equal parts by volume of polyethylene glycol 300 and water

In the study concerned with assessing the effect of concentration of CS on the irritant response it was demonstrated that as the concentration increased there was a tendency for the time to onset of initial sensation to become shorter and the total time of duration to become longer (fig 6) All subjects characteristically described the initial sensation to be stinging, and then developing into a detectable burning sensation, and which subsequently became strongly burning When the time to onset of each descriptive phase of sensation was plotted as a function of the concentration of CS applied to the tongue it was found that there was a tendency for the time to onset of each type of sensation to become shorter as the concentration increased (fig 7)

Discussion

Porszasz & Jancso (1959) noted that blepharospasm of the eye of the guinea pig was a reliable response to capsaicin We have found that the blepharospasm test is a relatively simple method which gives reproducible threshold values for irritancy with restricted 95 % confidence limits The blepharospasm test is easier to perform than other tests for irritancy, such as the guinea pig ileum or frog flexor reflex tests, since the preparation does not deteriorate, it uses an *in vivo* system, and individual variation is comparatively small in a given species In view of these considerations it is relatively easy to compare, and separate, irritant materials of even fairly closely related chemical structure Although the rabbit was found to be the least sensitive species

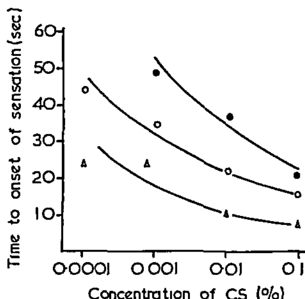


Fig 7 Effect of concentration of CS, dissolved in equal parts by volume of poly ethylene glycol 300 and water, on the time to onset of various types of sensation on the human tongue ● = strong burning ○ = detectable burning △ = stinging

to the blepharospasm-inducing properties of solutions of CS a satisfactory dose-response relationship was obtained The observations reported here have demonstrated that by using blepharopasm as basis for species comparison of threshold irritancy, the human eye is about 7 times more sensitive than the eye of the guinea pig and some 18 times more sensitive than the rabbit eye

The observations on the eye of man gave valuable information of a subjective nature not available from the animal studies Thus, whilst the EC50 for CS to produce blepharospasm was $3\,2\times10^{-6}$ M, that for sensation was $7\,3\times10^{-7}$ M In this way the sensitivity of the tests for initial signs of irritancy was increased by a factor of 4 5 using a subjective assessment If the grade 3 response line for sensation on the log probit plot (fig 4) is extrapolated, a 95 % incidence of grade 3 responses would be expected to occur at a concentration of $2\,7\times10^{-5}$M In the observations on man a score of 3 was close to the limit of tolerance for the subject Thus, a concentration of $2\,7\times10^{-5}$ M (i e 0 00051 % w/v) would be anticipated to be almost intolerable if applied to the eye and although the effect might be short lived at this concentration it would nevertheless be momentarily incapacitating This suggests that an expression of the data used to estimate EC50 values for irritancy in the form of a log-probit plot might be used as a guide for predicting completely incapacitating concentrations of an irritant material to the eye of man

Table 3

Threshold irritant response of the human eye to aerosols of CS

Concentration of CS (mg/m^3)	Response rate (1)
4.0×10^{-4}	1/10
4.7×10^{-4}	1/10
3.3×10^{-3}	2/10
4.0×10^{-3}	4/10
7.6×10^{-3}	8/10
1.9×10^{-2}	9/10
3.6×10^{-2}	10/10

EC50 (with 95 % confidence limits) $= 4.0\times10^{-3}$ $(2.3-6.6\times10^{-3})$

(1) Response rate recorded as $\frac{\text{number of positive responses}}{\text{Number of subjects tested}}$

It is of interest to compare the concentration of CS in solution required to produce threshold sensations on the human eye with the concentration of CS in the atmosphere required to elicit similar threshold effects. Table 3 gives the threshold concentrations of aerosols of CS required to produce stinging or burning sensations in the human eye (HOGG, personal communication) from which an EC50 of 4.0×10^{-3} mg CS/m^3 was calculated. The EC50 of solutions of CS to produce threshold effects was 7.3×10^{-7} M, i.e. 1.4×10^{-1} mg CS/l of solution compared with 4.0×10^{-6} mg CS/l of air for aerosols. These observations clearly indicate that the human eye is considerably more sensitive to CS in aerosol form than it is to solutions of CS. One major factor which probably influences the difference in response to aerosols as compared with solutions is the physical organization of the irritant material. In solution CS is present as a molecular dispersion, but in thermally generated aerosols it exists in a fine particulate form.

The EC50 for sensation on the human tongue was estimated at 6.8×10^{-6} M (0.00013 %), as compared with an EC50 of 7.3×10^{-7} M (0.000014 %) for sensation in the eye. Thus, the threshold concentration of CS required to elicit a sensation on the tongue is 9.4 times greater than that required to produce sensation in the eye. In the study concerned with defining any dose response relationship on the tongue, there appeared to be a definite decrease in the time to onset of each type of sensation (stinging or burning) and increase in the duration of sensation elicited with an increase in the concentration of CS. An important factor in measuring threshold concentrations appears to be the solvent system used to dissolve the irritant for test solutions. For example, in our observations using CS dissolved in normal

saline the median threshold value was estimated at 1 3 µg CS/ml, whereas CURRIE *et al* (1963) using CS dissolved in propylene glycol, recorded threshold concentrations between 0 25 and 0 5 µ CS/ml The estimation of threshold values for sensation on the tongue may have important practical uses For example, one consequence of the use of grenade generated smokes of CS in urban areas might be the contamination of foodstuffs in houses or shops Predetermined EC50 values for CS on the tongue give information about the levels of contamination that may be required to alter the taste of food, and more readily allow the design of experiments to determine the effects of contamination of food (KEMP & WILLDER 1972)

The estimation of EC50 values for irritancy is of value for comparing the relative potencies of different materials, providing an appropriate inert solvent is used and adhered to for all tests Although there are species differences in the response to a particular irritant material, the EC50 with a given species is highly reproducible Because of species variations, e g in the blepharospasm test, the results from animal observations cannot readily be extrapolated to man However, threshold studies for irritancy in man can be readily undertaken with little discomfort, and have the advantage that both the subjective and objective responses to an irritant may be measured Furthermore, in our tests man appeared to be more sensitive to an irritant than common laboratory mammals Before tests on irritancy to the eye of man are carried out, it is obviously necessary to determine that there is an adequate margin of safety between the concentrations to be used for irritancy tests and the lowest concentration of irritant which may cause even transient damage to the eye as determined by standard laboratory tests (BALLANTYNE & SWANSTON 1972, GRANT 1962, WELTMAN *et al* 1965) For example, in our tests using CS there was 6650 fold difference between the concentration likely to cause even just detectable transient damage to the cornea and the highest concentration used in the threshold studies (BALLANTYNE, unpublished results)

REFERENCES

Alarie Y Irritating properties of airbone materials to the upper respiratory tract. *Arch environ Health* 1966 **13** 433–449

Ballantyne B & S Callaway Inhalation toxicology and pathology of animals exposed to *o* chlorobenzylidene malononitrile (CS) *Med Sci Law* 1972 **12** 43–65

Ballantyne B & D W Swanston Ocular irritation tests *Brit J Pharmacol* 1972, **46** 577–578 P

Currie H, L P Chenier & W C Stewart Sensory effects of CS on the tongue of man *Suffield Technical Paper No 286* October 1963 Suffield Experimental Station Canada.

Finney, D J *Probit analysis* Cambridge University Press London 1962

Grant, W M *Toxicology of the eye* Charles C Thomas Springfield 1962

Himsworth Report 1971 Report of the enquiry into the medical and toxicological aspects of CS (orthochlorobenzylidene malononitrile) Part 2 Enquiry into toxicological aspects of CS and its use for civil purposes Her Majesty's Stationery Office, Command 4775

Keele C. A The common chemical sense and its receptors *Arch int Pharmacodyn Ther* 1962 139, 547–557

Kemp K. H & W B Willder The palatability of food exposed to *o* chlorobenzylidene malononitrile (CS) *Med Sci Law* 1972 12, 113–120

Owens E J & C L. Punte Human respiratory and ocular irritation studies utilizing *o*-chlorobenzylidene malononitrile aerosols *Amer Industr Hyg Ass J* 1963, 24, 262–264

Porszasz, J & N Jancso Studies on the action potentials of sensory nerves in animals desensitised with capsaicine *Acta physiol Hungarica* 1959, 16 299–306

Punte C L., P J Gutentag E J Owens & L. E Gongwer Inhalation studies with chloracetophenone, diphenylaminochloroarsine and pelargonic morphilide *Amer Industr Hyg Ass J* 1962 23 199–202

Weltman A S, S B Sparber & T Jurtshuk Comparative evaluation and the influence of various factors on eye irritation scores *Toxicol Appl Pharmacol* 1965, 7, 308–319

Acta pharmacol et toxicol 1973, 32, 278–284

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Anti-Nociceptive Activity of Some Thiophthalanes with Morphine-like Properties

By

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Abstract The anti nociceptive activity of a series of thiophthalanes and some related compounds has been compared to that of five known anti nociceptive drugs Among the series several compounds were found to possess typical morphine like properties They were as active as morphine in various anti nociceptive tests in mice, produced a Straub Herrmann tail reaction and their anti nociceptive activity was antagonized by nalorphine The most potent compound of this series was 3 3 dimethyl 1 dimethylaminoethyl 1 phenylthiophthalane (Lu 6-062) The corresponding sulphoxide and the sulphone were slightly less active, but showed a faster onset of effect Thus it is suggested that the effect of Lu 6 062 may be mediated through the sulphoxide metabolite

Key words Analgesia – pharmacology – morphine – thiophthalanes

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A series of bicyclic compounds with thymoleptic properties has previously been described from this laboratory (PETERSEN *et al* 1966 & 1969) These were all compounds with a propylamino side chain Screening of a new series of compounds with a similar ring structure, but with an ethylamino side chain, revealed that some of the compounds had quite potent anti-nociceptive activities in mice

Further studies indicated that some of the drugs showed morphine like properties

Since drugs of this chemical configuration have not previously been shown to possess morphine-like activities, it was decided to carry out a study with the purpose of clarifying the optimal chemical configuration in this respect

The general structure of the compounds studied is given in fig 1

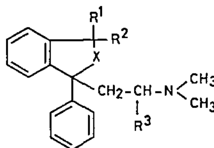


Fig 1 General structure of the compounds studied

Material and Methods

In all tests male mice, NMRI/BOM, SPF, 18–25 g were used

Hot plate test

The mice were placed in a perspex cylinder on a copper plate heated to 58°. Normally the animals react to this heat stimulus by lifting their paws, licking their paws or jumping out of the cylinder Three to four groups of animals (10 animals per group) were tested before treatment and their reaction times were recorded

After the injection of the test compound the animals were retested at 15, 30, 45 and 60 minutes respectively The ED50 was determined as the dose that abolished the reaction in a test period of 30 seconds

Tail pinching test

A modification of the method described by HAFFNER (1929) was used Before the experiments the animals were pinched at the root of the tail with a pair of forceps Squeaking or biting after the forceps was considered as a positive reaction If the animals did not react positively, they were not included in the experiment The test compound was injected intraperitoneally at 3 or 4 dose levels with 10 animals per group using only the positive reacting mice

Each mouse was rested 15, 30, 45 and 60 minutes respectively, after drug administration The ED50 was the dose which prevented squeaking or biting in 50 % of the animals

Electrical tail stimulation

A modification of the method described by LUND NIELSEN (1961) was used Mice were placed in narrow wire mesh cylinders and two needle electrodes, 8 mm apart, were placed subcutaneously at the root of the tail Electrical stimuli (square wave 1 Hz, 20 msec, 4 sec) were applied through the electrodes with an increasing voltage until a squeak was heard The voltage required for this reaction was defined as the threshold The threshold of untreated animals varied from 2 to 8 volts The test compound was administered intraperitoneally at 3–4 dose levels with 10 animals per group After the injection of the test compound the threshold was redetermined at 15, 30 45 and 60 min utes The ED50 was the dose which doubled the threshold in 50% of the animals

Straub Herrmann tail reaction

All the mice were observed for the occurrence of the Straub Herrmann tail reaction

Table 1

Structure and pharmacological results of the compounds studied

Serial No	Code No	X	R1	R2	R3	ED50 mg/kg Hot plate s c	Tail stimulation i p	Tail pinch i p	Straub-Herrmann tail
1	Lu 6–062	S	CH_3	CH_3	H	13	4 5	9	+
2	Lu 7–065	SO	CH_3	CH_3	H	42	10	19	+
3	Lu 6–087	SO_2	CH_3	CH_3	H	9 5	5	10	+
4	Lu 3–090	C	CH_3	CH_3	H	> 80			–
5	Lu 3–042	O	CH_3	CH_3	H	> 40	10	30	+
6	Lu 10–023	S	CH_3	H	H	> 40			–
7	Lu 10–024	SO	CH_3	H	H	> 40			–
8	Lu 9–225	S	H	H	H	> 20	8	25	(+)
9	Lu 10–009	SO	H	H	H	> 80			–
10	Lu 9–231	O	H	H	H	> 20	4.5	15	(+)
11*	Lu 6–076	S	CH_3	CH_3	CH_3	56	7	29	+
12*	Lu 6–088	S	CH_3	CH_3	CH_3	39	8 5	12	+
13	Lu 10–028	SO	CH_3	CH_3	CH_3	38	11	14	+
14	Lu 7–064	O	O		H	> 80	3	25	(+)
15	Morphine					15	3	18	+
16	Methadone					8	2 5	8	+
17	Ketobemidone					7 5	2.5	4	+
18	Pethidine					30	12	29	+
19	Dextropropoxyphene					48	14	46	(+)

* Serial Nos 11 and 12 Isomers

(Herrmann 1912) The results given in table 1 refer to the presence + or absence – of this reaction in analgesic doses

Chemical

The compounds studied are given in table 1 The compounds were prepared as water soluble hydrochlorides and the results given in the following refer to weighed amounts of these substances

Results

In table 1 the compounds are identified by code numbers or generic names for the reference substances In order to facilitate reference to any one compound they have also been assigned a serial number (column 1) The results of the pharmacological experiments are also listed in table 1 It

appears that the compounds Lu 6-062 and Lu 6-087 (serial Nos 1 and 3) are as active as morphine (15) Lu 3 042 (5) which is the phthalane analogue of Lu 6-062 (1) showed relatively weak activity The thiophthalanes Lu 7 065 Lu 6 076 Lu 6-088 and Lu 10-028 (2 11 12 and 13) possess anti nociceptive activities comparable to those of pethidine (18) and dextropropoxyphene (19) All the compounds mentioned so far caused a Straub-Herrmann tail reaction Most of the other compounds studied had only weak anti nociceptive activities as compared to the reference substances and did not cause a Straub-Herrmann tail reaction the exception being compounds Lu 9 225 Lu 9 231 and Lu 7-064 (8 10 and 14) which showed weak Straub-Herrmann tail reactions

It is well known that the anti nociceptive activity of morphine like drugs can be abolished by nalorphine (GRUMBACH & CHERNOV 1965 MARTIN 1967) and the demonstration of a nalorphine antagonism has been used as a test for morphine like activity (JANSSEN 1961)

The antagonistic effect of nalorphine against Lu 6 062 (1) and morphine (15) has been studied using the hot plate test The anti nociceptive activity of both morphine (15) and Lu 6 062 (1) was completely abolished by simultaneous treatment with nalorphine (20 mg/kg subcutaneously)

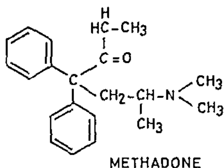
Discussion

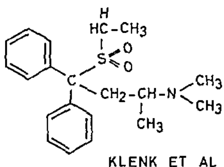
Thiophthalanes with a 3 carbon atom side chain have been shown in our laboratories not to possess anti nociceptive activity and not to cause a Straub-Herrmann tail reaction Thus the active compounds are found among those with 2 C atoms between the central carbon atom and the amino group

Compounds in which X is S SO or SO_2 were the most potent analgesics The phthalanes (X=O) were considerably less active (5 and 10) The phthalide No 14 was about equipotent with the phthalanes The indane No 4 ($X=CH_2$) did not possess any anti nociceptive activity

Compounds in which one or both of the substituents R^1 and R^2 were H showed weaker activity (6 7 8 9 and 10) than the corresponding dimethylated compounds

The compounds described in this paper may be regarded as ring closed analogues of analgesics of the methadone-type (fig 2) These latter analgesics require for activity a quarternary substituted carbon atom connected to 1) two aromatic rings 2) a two carbon side-chain with a tertiary amino group at the β carbon atom and 3) an electro-negative group The electro negative group may be an alkylketo group as in methadone but a marked analgesic activity has also been reported for the alkylsulphone analogues (KLENK *et al* 1948) The optimal alkyl group with regard to analgesic activity in both





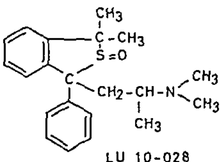


Fig 2 Structural comparison of methadone and sulphone analogues of methadone with Lu 10-028

series of methadone-like substances is ethyl Methylketones and -sulphones still exhibit some activity whereas substances with other alkyles are almost completely devoid of activity (WALTON *et al* 1949) This is in contrast to our ring closed compounds where the most active are those with an isopropyl group

The thiophthalanes showing anti-nociceptive activity (1, 11 and 12) have no electro-negative group at the central carbon atom It is, however, possible that these compounds are not active *per se*, but only after oxidation to the

sulphoxides This is indicated by the fact that the thiophthalanes have a slower onset of action than the corresponding sulphoxides and sulphones Thus, Lu 6 062 (X=S) showed a maximal effect 60 min after the injection whereas the peak of effect for Lu 7-065 (X=SO) and Lu 6-087 ($X=SO_2$) occurred 15 min after injection Oxidation of the thiophthalanes or their sulphoxides to the sulphones is unlikely since studies on the metabolism of a chemically related thiophthalane, Lu 5-003 (3,3-Dimethyl-1-(3-methylaminopropyl)-1-phenylthiophthalane) have shown that the sulphoxide was the main metabolite and that oxidation to the sulphone did not occur at all (FREDRICSON OVERØ *et al* 1970)

In the methadone series substitution by a methyl group at the carbon atom α to the amino group in the side chain results in increased activity (KLENK *et al* 1948) This does not seem to be the case in our series of compounds Thus, the two sulphoxides Lu 7-065 (2) and Lu 10-028 (13) are about equipotent, and the thiophthalane derivatives Lu 6 076 (11) and Lu 6-088 (12) with an α-methyl group in the side chain are less active than Lu 6 062 (1) These findings may indicate that a methyl group in the α position of the side chain inhibits sulphoxidation of the thiophthalanes

In conclusion the compounds presented in this paper represent a hitherto unknown chemical configuration possessing morphine-like activity In many respects, however, the configuration of the new compounds is in agreement with the general lines for chemical structure of synthetic morphine-like analgesics, especially if the hypothesis holds that the very active thiophthalane derivatives have to be converted into active metabolites in order to exert their anti-nociceptive effect

REFERENCES

Fredricson Overø, K, A Jørgensen & V Hansen Metabolism distribution and excretion of the thiophthalane Lu 5-003 a bicyclic thymoleptic *Acta pharmacol et toxicol* 1970 28, 81–96

Grumbach, L & H I Chernov The analgesic effect of opiate opiate antagonist combinations in the rat *J Pharmacol Exp Therap* 1965 149 385–396

Haffner F Experimentelle Prufung schmerzstillender Mittel *Deutsch Med Wsch* 1929 55 731–732

Herrmann, O Eine neue Nachweismethode des Morphins *Biochemische Zeitschr* 1912 39 216–219

Janssen P A J Pirinitramide (R 3365) a potent analgesic with unusual chemical structure *J Pharm Pharmacol* 1961 13 513–530

Klenk, M M, C M Suter & S Archer The preparation and properties of some benzohydrylsulfones *J Amer Chem Soc* 1948, 70 3846–3850

Lund Nielsen P Studies on algesimetry by electrical stimulation of the mouse tail *Acta pharmacol et toxicol* 1961 18 10–22

Martin W R Opioid antagonists *Pharmacol Rev* 1967 19 463–521

Petersen, P V, N Lassen, T Ammitzbøll, I Møller Nielsen, M Nymark, V Pedersen & K F Franck Further pharmacological studies of bicyclic thymoleptics *Acta pharmacol et toxicol* 1970 **28**, 241–248

Petersen P V, N Lassen V Hansen, T Huld, J Hjortkjær, J Holmblad I Møller Nielsen, M Nymark, V Pedersen A Jørgensen & W Hougs Pharmacological studies of bicyclic thymoleptics *Acta pharmacol et toxicol* 1966, **24**, 121–133

Walton E, P Ofner & R H Thorp Search for new analgesics Part III Homologues of amidone, isoamidone, and some related compounds *J Chem Soc* 1949, **1**, 648–655

Acta pharmacol et toxicol 1973, 32, 285–288

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Azaperone and the Hepatic Microsomes: Effects on Cytochrome P-450 Concentration and on NADPH-Cytochrome c-Reductase Activity

By

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(Received March 22, 1972, Accepted September 6, 1972)

Abstract Twelve male mice weighing about 16 g were given 4 mg/kg azaperone subcutaneously in 0.5 ml of water Twelve similar mice which were given 0 5 ml of water subcutaneously at the same time were kept as controls After 7 days all the mice were sacrificed and the total protein content, the microsomal protein content the microsomal cytochrome P-450 concentration and the microsomal NADPH-cytochrome c reductase activity of the livers was determined In the azaperone treated group the mean microsomal cytochrome P 450 concentration of the livers was significantly increased but the mean microsomal NADPH-cytochrome c reductase activity of the livers was significantly decreased as compared to the controls

Key words Azaperone – cytochrome P 450 – NADPH cytochrome c reductase activity – liver microsomes

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The administration to experimental animals of certain drugs, such as phenobarbital (phenemalum NFN), results in an enhanced metabolic rate in the liver microsomes, this change affects not only the drug administered, but also numerous other unrelated compounds (Conney *et al* 1960) In addition to with the increased rate of metabolism, an increase in cytochrome P-450 content and in NADPH cytochrome c reductase activity is observed (Ernster & Orrenius 1965) Such drugs often also increase the total protein and microsomal protein content of the liver (Conney *et al* 1960)

Azaperone, (stresnil, 40 mg azaperone/ml, Janssen Pharmaceutica N V, Beerse, Belgium) 4 -fluoro-4-[4 (2 pyridyl)-1 piperazinyl] butyrophenone, is a new neuroleptic used as a sedative for pigs The purpose of this investigation was to study the effect of a single azaperone administration on the total protein content, on the microsomal protein content, on the micro somal cytochrome c-reductase activity and on the microsomal cytochrome P-450 content of the liver in mice

Material and Methods

Male NMRI mice (24) weighing about 16 g (range 15 1–17 4 g) were divided into two equal groups Group 1 received azaperone in a dose of 4 mg/kg, subcutaneously in about 0.5 ml water At the same time group 2 was given 0.5 ml water subcutaneously Before and during the experiment, the mice were fed a standard laboratory diet *ad libitum* (Orion, Helsinki, Finland) After 7 days all the mice were weighed and sacrificed under light ether anaesthesia by cutting off the abdominal aorta. The livers were rapidly removed and after being weighed a 10 % (w/v) suspension was made in an ice-cold homogenizing medium consisting of 0 25 M sucrose in 0 05 M potassium phosphate buffer, pH 7 4 The homogenisation was performed with a Thomas tissue grinder (A. H Thomas, Philadelphia, USA) for 30 seconds and with 10 up and down movements with the pestle The vessel was kept in ice water during the homogenisation. One ml aliquot of this homogenate was used for the total protein determination (LOWRY *et al* 1951)

The remainder of the homogenate was then centrifuged for 10 minutes at 12 000 × g to remove mitochondria and other cellular debris, and a second 12 000 × g centrifugation was performed to ensure the elimination of mitochondria The post mitochondrial supernatant was centrifuged for 45 minutes at 78 000 × g The microsomal pellet collected was resuspended in 0 125 M KCl and 0 05 M potassium phosphate buffer, pH 7 4 and recentrifuged for another 30 minutes at 78,000 × g The resulting pellet was suspended in 0 1 mM EDTA and 0 05 M potassium phosphate buffer, pH 7 7 Microsomal protein was determined according to LOWRY *et al* (1951)

NADPH cytochrome c-reductase was assayed according to the method of MASTERS *et al* (1967) The cytochrome P-450 concentration was determined from the CO difference spectrum of dithionite-reduced microsomes (OMURA & SATO 1964) using a Beckman DB G dual beam spectrophotometer and quartz cuvettes (1 cm light path)

Results

The results of the total liver protein determinations, of the liver microsomal protein determinations and of the assay of NADPH-cytochrome c-reductase activity and the cytochrome P-450 content of the liver microsomes of the animals are presented in table 1 The mean increase in weight of the mice or the mean weight of the livers did not significantly differ between the azaperone-treated and the control animals

Discussion

The mean total liver protein and the mean microsomal protein were not significantly affected by the treatment with azaperone The mean cytochrome P-450 concentration increased significantly ($P < 0\ 01$) in the azaperone-treated group as compared to the controls In this respect, then, azaperone has properties similar to those of several known stimulators of microsomal

Table 1.

The effect of azaperone treatment on the total protein content, on the microsomal protein content, on the microsomal cytochrome P-450 concentration and on the microsomal NADPH cytochrome c reductase activity in the liver of mice

Mice	Total liver protein, mg/g liver	Microsomal protein, mg/g liver	Cytochrome P-450 concentration, CO difference spectra*) (x10³)	NADPH cytochrome c-reductase activity**)
Control	123.50 ± 6 12 ***)	68.37 ± 8.30	29 6 ± 7.2	20 1 ± 3 4
	n 12	n 11	n 11	n 11
Azaperone-treated	127 00 ± 7 85	72 46 ± 7 64	43 6 ± 4 4	16 9 ± 2 6
	n 11	n 11	n 11	n 11
	P < 0 4	P < 0 4	P < 0 01	P < 0 05

*) △ O D 450–500 nm × mg⁻¹ microsomal protein
**) nmol × min⁻¹ × mg⁻¹ microsomal protein
***) mean ± s
n number of animals

drug metabolism (CONNEY *et al* 1960, CONNEY 1967, SLADEK & MANNERING 1969)

The mean NADPH-cytochrome c-reductase activity was found to be significantly (P < 0 05) decreased in the liver microsomal suspensions of the azaperone-treated mice as compared to controls *In vitro* experiments with hepatic microsomes from male rats have shown that several drugs known as inducers of drug metabolism are without effect or decrease the activity of NADPH-cytochrome c-reductase (GIGON *et al* 1969) It has also been shown that NADPH-cytochrome c-reductase activity does not correlate well with oxidative drug metabolism in animals (DAVIES *et al* 1969) Therefore the inhibition of NADPH-cytochrome c-reductase activity observed in the microsomal suspension of the azaperone-treated mice does not necessarily have any correlation to the rate of drug metabolism, but obviously provides information as to the metabolic mechanism of the drug in the hepatic microsomes as suggested by GIGON *et al* (1969)

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REFERENCES

Conney, A H, C. Davison, R Gastel & J J Burns Adaptive increases in drug metabolizing enzymes induced by phenobarbital and other drugs *J Pharmacol exp Therap* 1960, **130**, 1-8

Conney, A H Pharmacological implications of microsomal enzyme induction *Pharmacol Rev* 1967, **19**, 317-366

Davies, F I, P L. Gigon & J R Gillette Species and sex differences in electron transport systems in liver microsomes and their relationship to ethylmorphine demethylaction *Life Sci* 1969, **8**, 85-91

Ernster, L & S Orrenius Substrate induced synthesis of the hydroxylating enzyme system of liver microsomes *Fed Proc* 1965, **24**, 1190-1199

Gigon, P L, T E Gram & J R Gillette Studies on the rate of reduction of hepatic microsomal cytochrome P-450 by reduced nicotinamide adenine dinucleotide phosphate Effect of drug substrates *Mol Pharmacol* 1969, **5** 109-122

Lowry, O H, N J Rosebrough, A L Farr & R J Randall Protein measurement with the folin phenol reagent *J Biol Chem* 1951, **193**, 265-275

Masters, B S S, C H Williams & H Kamin The preparation and properties of microsomal TPNH cytochrome c reductase from pig liver In *Methods of enzymology*, vol X Ed R W Estabrook and M E Pullman Academic Press, New York, 1967, pp 565-573

Omura, T & R Sato The carbon monoxide binding pigment of liver microsomes II Solubilisation, purification and properties *J Biol Chem* 1964, **239**, 2379-2385

Sladek, N E & G J Mannering Induction of drug metabolism 1 Differences in the mechanisms by which polycyclic hydrocarbons and phenobarbital produce their inductive effects on microsomal N demethylating systems *Mol Pharmacol* 1969 **5**, 174-185

Acta pharmacol et toxicol 1973, 32, 289–293

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Inductive Effects of Methyl Mercury on the Hepatic Microsomes of Mice

By

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(Received April 17, 1972, Accepted September 6, 1972)

Abstract 36 male NMRI mice weighing about 17 g each were divided into groups of 12 animals Groups 1 and 2 were treated once intraperitoneally with methyl mercury nitrate Group 1 received 0 3 mg Hg/kg and group 2 1 0 mg Hg/kg in 0 5 ml water, the third group, which was kept as control, was treated intra peritoneally with 0 5 ml water After 7 days all the mice were sacrificed The mean total liver protein was found to be significantly decreased in group 2 The mean liver microsomal protein was significantly ($P < 0.01$) increased in groups 1 ($P < 0.01$) and 2 ($P < 0.02$) as compared to the controls The mean cytochrome P 450 concentration of the liver microsomes was also significantly increased in groups 1 ($P < 0.001$) and 2 ($P < 0.01$) as compared to the controls and in group 1 ($P < 0.01$) as compared to group 2 The mean liver microsomal NADPH-cytochrome c reductase activity was significantly greater in group 2 ($P < 0.001$) and in group 1 ($P < 0.01$) than in the controls The mean NADPH cytochrome c-reductase activity of group 2 was also significantly greater than the mean activity in group 1 ($P < 0.02$)

Key words Mercury – microsomes – liver

Previous work has shown that the pretreatment of rats with methyl mercury shortens the duration of hexobarbital (enhexymalum NFN) hypnosis (PEKKANEN & PEKKARINEN 1972) This indicates that the properties of methyl mercury resemble those of some other compounds, such as DDT and phenobarbital (phenemalum NFN) these compounds are known as inducers of hepatic microsomal drug metabolism, which similarly shorten the duration of hexobarbital hypnosis (CONNEY *et al* 1960) In addition to the increased rate of metabolism in the hepatic microsomes caused by such inducers there is an increase in the microsomal cytochrome P-450 content and in the microsomal NADPH-cytochrome c-reductase activity (ERNSTER & ORRENIUS 1965) Such drugs also increase the total protein and microsomal protein content of the liver (CONNEY *et al* 1960)

The purpose of this investigation was to study the effect of methyl mercury

treatment on the total protein content, on the microsomal protein content, on the microsomal cytochrome P-450 content and on the microsomal cytochrome c-reductase activity of the liver of mice

Material and Methods

Male NMRI mice (36) weighing about 17 g (range 14 1–20 1 g) were divided into three equal groups Group 1 received methyl mercury nitrate in a dose of 0 3 mg Hg/kg intraperitoneally in about 0 5 ml water, group 2 was similarly given methyl mercury nitrate in a dose of 10 mg Hg/kg and group 3, the control group, was given 0 5 ml water intraperitoneally Before and during the experiment the mice were fed a standard laboratory diet *ad libitum* (Orion, Helsinki, Finland) After 7 days all the mice were weighed and sacrificed under light ether anaesthesia by severing the abdominal aorta. The livers were rapidly removed and after they were weighed a 10 % (w/v) suspension was made in an ice cold homogenizing medium consisting of 0 25 M sucrose in 0 05 M potassium phosphate buffer, pH 7 4 The homogenisation was performed with a Thomas tissue grinder (A H. Thomas, Philadelphia, USA) for 30 seconds and with 10 up and down movements with the pestle The vessel was kept in ice water during the process of homogenisation One ml aliquot of this homogenate was used for the total protein determination (LOWRY *et al* 1951)

The remainder of the homogenate was then centrifuged for 10 minutes at 12,000 × g to remove mitochondria and other cellular debris, and a second 12 000 × g centrifugation was performed to ensure elimination of mitochondria The post mitochondrial supernatant was then centrifuged for 45 minutes at 78 000 × g The microsomal pellet collected was resuspended in 0 125 M KCl and 0 05 M potassium phosphate buffer, pH 7 4 and recentrifuged for an other 30 minutes at 78,000 × g The resulting pellet was suspended in 0 1 mM EDTA and 0 05 M potassium phosphate buffer pH 7 7 Microsomal protein was determined according to LOWRY *et al* (1951)

NADPH cytochrome c reductase was assayed according to the method of MASTERS *et al* (1967) Cytochrome P 450 concentration was determined from the CO difference spectrum of dithionite reduced microsomes (OMURA & SATO 1964) using a Beckman DB G dual beam spectrophotometer and quartz cuvettes (1 cm light path)

Results

The results of the total liver protein determinations, of the liver microsomal protein determinations and of the assay of NADPH cytochrome c-reductase activity and the cytochrome P-450 content of the liver microsomes of the animals are presented in table 1 The mean increase in weight of the mice during the course of the experiment in group 1 was 6 8±2 3 g in group 2 2 6±1 9 g and in the control group 5 2±2 0 g The differences between the means are not significant The mean weight of the livers of the mice in group 1, expressed in per cent of body weight, is 5 26±0 42 %, which does not differ significantly from the corresponding mean of the controls, 5 84±0 65 %, and of group 2, in which the percentage was 5 61±0 54 %

Table 1

The effect of methyl mercury treatment on the total protein content, on the microsomal protein content, on the microsomal cytochrome P-450 concentration and on the microsomal NADPH cytochrome c reductase activity in the liver of the mouse
Group 1 was given intraperitoneally 0.3 mg Hg/kg and group 2 10 mg Hg/kg All the mice were sacrificed 7 days after the methyl mercury treatment

Mice	Total liver protein, mg/whole liver	Microsomal protein, mg/g liver protein	Cytochrome P-450 concentration, CO difference spectra*) (×10³)	NADPH cytochrome c reductase activity**)
Control	156 07 ± 19 93***) n 12	68 37 ± 8 30 n 12	29 5 ± 7 2 n 11	20 1 ± 3 4 n 12
Group 1 (0 3 mg Hg/kg)	153 56 ± 18 18d) n 12	83.20 ± 9 96 b) n 9	49 3 ± 3 7 a) d) n 12	29 0 ± 4 3 b) e) n 9
Group 2 (10 mg Hg/kg)	133 13 ± 22 15 b) d) n 12	79 88 ± 8,48 c) n 8	35 0 ± 7 2 b) d) n 10	34 1 ± 6 2 a e) n 8

Δ O D 450–500 nm × mg ¹ microsomal protein
nmol × min ¹ × mg⁻¹ microsomal protein
) mean ± s
number of animals
P < 0 001 as compared to the controls
P < 0 01 as compared to the controls
P < 0 02 as compared to the controls
P < 0 01 as compared to the other CH_3Hg treated group
P < 0 02 as compared to the other CH_3Hg treated group

Discussion

The mean total liver protein (mg/whole liver) was significantly ($P < 0.01$) smaller in group 2 than in group 1 or in the controls. This indicates that the total protein synthesis was inhibited in the liver of mice treated with methyl mercury at the 10 mg Hg/kg level. The smaller methyl mercury dose of 0.3 mg Hg/kg did not significantly affect the total protein content of the livers as compared to the controls. The mean microsomal protein content, mg/g liver protein, was significantly increased in both methylmercury treated groups (group 1 at level $P < 0.01$ and group 2 at level $P < 0.02$), as was also the mean liver microsomal cytochrome P-450 concentration per mg microsomal protein as compared to the corresponding means of the controls (group 1 $P < 0.001$ and group 2 $P < 0.01$). This indicates that methyl mercury in this respect resembles several other drugs known as inducers of microsomal drug metabolism (CONNEY *et al* 1960, ERNSTER & ORRENIUS 1965). The mean liver microsomal cytochrome P-450 concentration per mg microsomal protein was significantly ($P < 0.01$) greater in group 1, which was treated with a smaller dose of methyl mercury (0.3 mg Hg/kg), than in group 2, treated with a greater dose of methyl mercury (10 mg Hg/kg). Together with an increase in the liver microsomal protein content and an increase in the liver microsomal cytochrome P-450 concentration, an increase in the rate of drug metabolism was also observed (CONNEY *et al* 1960, ERNSTER & ORRENIUS 1965). Therefore at the lower dose level the metabolic rate of methyl mercury should obviously be greater than at the higher dose level. This has actually been shown by ÖSTLUND (1969) who observed that the biological half-life of methyl mercury in mice increases significantly with an increasing dose level, although he was unable to point to the mechanisms responsible for this.

The mean NADPH cytochrome c reductase activity of liver microsomes was significantly (group 1 $P < 0.01$, group 2 $P < 0.001$) increased in both methyl-mercury treated groups as compared to the controls, and the mean activity was significantly ($P < 0.02$) greater in group 2 as compared to group 1. Although the NADPH-cytochrome c reductase activity does not correlate well with the rate of oxidative drug metabolism in animals (DAVIES *et al* 1969), the increase in the mean liver microsomal NADPH-cytochrome c reductase activity observed in the methyl-mercury treated animals provides information as to the metabolic mechanism of methyl mercury in the hepatic microsomes as suggested by GIGON *et al* (1969).

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REFERENCES

Conney, A H, C. Davison, R Gastel & J J Burns Adaptive increases in drug metabolizing enzymes induced by phenobarbital and other drugs *J Pharmacol exp Therap* 1960, 130, 1–8

Davies, F J, P. L. Gigon & J R Gillette Species and sex differences in electron transport systems in liver microsomes and their relationship to ethylmorphine demethylaction *Life Sci* 1969, 8, 85–91

Ernster, L & S Orrenius Substrate induced synthesis of the hydroxylating enzyme system of liver microsomes *Fed Proc* 1965, 24 1190–1199

Gigon P L., T E Gram & J R Gillette Studies on the rate of reduction of hepatic microsomal cytochrome P 450 by reduced nicotinamide adenine dinucleotide phosphate Effect of drug substrates *Mol Pharmacol* 1969, 5, 109–122

Lowry, O H, N J Rosebrough, A L Farr & R J Randall Protein measurement with the folin phenol reagent *J Biol Chem* 1951, 193 265–275

Masters, B S S, C H Williams & H Kamin The preparation and properties of microsomal TPNH cytochrome c reductase from pig liver In *Methods of enzymology*, vol X Ed R W Estabrook and M E Pullmann Academic Press, New York, 1967, pp 565–573

Omura, T & R Sato The carbon monoxide binding pigment of liver microsomes II Solubilisation, purification and properties *J Biol Chem* 1964, 239, 2379–2385

Pekkanen, T & K Pekkarinen The effect of methyl mercury pretreatment on the duration of hexobarbital hypnosis on rat *Acta vet scand* 1972, 13, 149–150

Östlund, K Studies on the metabolism of methyl mercury and dimethyl mercury in mice *Acta pharmacol et toxicol* 1969, 27, Suppl 1, 57–62

Acta pharmacol et toxicol 1973, 32 294–303

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Drug Infusion into the Spinal Subarachnoid Space of Unanaesthetized Rats during Early Postnatal Life: A Technique for the Study of Brain Development

By

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(Received June 29, 1972, Accepted September 21, 1972)

Abstract A convenient and rapid method for percutaneous catheterization of the spinal subarachnoid space of rats is described The technique is particularly suited for the study of brain development and enables the investigator to infuse drugs intrathecally into unanesthetized animals during early postnatal life At the termination of a five minute infusion of 40 and 90 microliters of Evans blue dye to five and thirty day old rats respectively, complete penetration of the subarachnoid space and ventricular system is found A similar infusion of carboxyl ^{14}C inulin to thirty day old animals results in an immediate and equal distribution of radioactivity to both sides of the brain In five day old rats a similar symmetrical distribution occurs within 30 minutes In both groups of animals a three fold elevation of spinal fluid pressure is found immediately after infusion which rapidly decreases to normal resting levels within 10 minutes. In contrast to earlier cerebroventricular injection techniques the present method avoids the problem of unequal distribution gradients created when drugs are infused near the periventricular region

Key words Intrathecal infusion – carboxyl ^{14}C inulin – brain development

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The extracellular spaces in the brains of adult rodents are of ample size for the free diffusion of relatively large molecules (BRIGHTMAN 1968) These spaces are even larger in newborn rat and decrease in size to reach adult dimensions by 14–16 days of postnatal life (CALEY & MAXWELL 1970) The extracellular fluid of the brain appears to be in equilibrium with the cerebrospinal fluid in the ventricles and cisterna magna, and although not chemically identical, these fluids are very similar in composition (AMES *et al* 1964, BITO & DAVSON 1966, OLENDORF & DAVSON 1967, RALL 1968) Large molecular weight substances, such as inulin and horseradish peroxidase, have been shown to readily diffuse from the ventricular fluid through brain parenchyma via the extracellular space (RALL *et al* 1962, BRIGHTMAN 1968) Hence, it

would appear that the ependymal and pial linings exert little restraint on the penetration of substances into the brain, resulting in a continuous flux of solutes between the cerebrospinal and extracellular fluids

In the present study, a technique is described for the infusion of drugs into the cerebrospinal fluid system of the unanaesthetized rat during early postnatal life The dye Evans blue was found to penetrate completely into the ventricular system and subarachnoid space, and tracer doses of carboxyl-^{14}C inulin were shown to be distributed symmetrically on both sides of the brain Hence, the method allows the direct administration of substances into the extracellular spaces of the brain for studying the effects of drugs on differentiating neural cells

Materials and Methods

Eighty Sprague-Dawley strain albino rats from eight litters were used Eight pregnant female rats at 14–16 days of gestation, were obtained and fed *ad libitum* on a standard laboratory rodent diet Parturition occurred on day 21 to 23 At 5 and 30 postnatal days of age animals were divided into two groups of 40 rats each

Lumbar puncture Five and 30 day old rats were lightly sedated with chloroform and in the prone position immobilized by gauze wrapping placed over their head and upper body The vertebral column just above the iliac crest was firmly grasped between the thumb and index finger and fixed into position A no 15 gauge spinal needle (3 centimeters in length) was then inserted through the skin and sustained pressure exerted directly in line with the vertebral column at the L4-5 level This resulted in penetration of the dorsal vertebral arch and entry of the needle into the spinal canal The depth of penetration never exceeded 3–5 millimeters The procedure produced an immediate flaccid paralysis and complete loss of pain sensation in the hind limbs and tail secondary to traumatic injury of the spinal cord

Catheterization A polyethylene catheter (outer diameter – 0 8 mm inner diameter – 0 4 mm Portex PP 25) was cut to a length of 7 cm for 30 day old and 5 cm for 5 day old rats so calculated as to allow the tip of the catheter to lie at the T3-4 vertebral level The volume of the "dead space" of the catheter (2 microliters per centimeter of length) was determined with a microsyringe fitted with a Chaney adaption (Hamilton Company, Whittier California) The catheter was then threaded through the lumen of the needle into the spinal canal Attempts to pass the catheter higher than the mid thoracic level should be avoided for although the cisterna magna may be entered via this route, the catheter may perforate the medulla resulting in immediate respiratory distress and death Misdirection of the catheter secondary to faulty placement of the spinal needle results in either (1) an inability to pass the catheter because of soft tissue resistance or (2) an excessive ease of passage into the peritoneal cavity which is confirmed by palpation of the catheter through the abdomen The catheter when correctly placed always occupied the subarachnoid space, and occasionally a flow of spinal fluid and venous pulsations were observed No mortality resulted from the catheterization, and long term survival with special paraplegic care of the animal could be obtained

Intrathecal infusion All infusions were performed after the animals had completely recovered from light chloroform sedation and without the use of anaesthetics or im

mobilizing agents The catheter was attached to a one milliliter syringe mounted on an infusion pump The infusion rate was calculated to deliver 40 microliters at a rate of 11 2 microliters per minute, and 90 microliters at a rate of 20 4 microliters per minute to 5 and 30 day old animals respectively

Solutions containing 1 % Evans blue (Merck Chemical Co) and carboxyl ^{14}C inulin (3 05 mci/gram, molecular weight 5000–5500, New England Nuclear Corp) were made using a diulent with a composition similar to that of cerebrospinal fluid (NaCl – 7 0 g/l $NaHCO_3$ – 1 9 g/l, NaH_2PO_4 H_2O – 0 014 g/l, Na_2HPO_4 – 0 057 g/l, KCl – 0 268 g/l $MgSO_4$ – 0 145 g/l $CaCl_2$ – 0 128 g/l, Glucose – 1 8 g/l, Solution adjusted to pH 7 4 by titration using concentrated HCl) Forty microliters of either a 1 % solution of dye or 191 nanocuries of carboxyl ^{14}C inulin was infused into the spinal subarachnoid space of 5 day old rats Similarly, ninety microliters of a 1 % solution of dye or 385 nanocuries of carboxyl ^{14}C inulin was infused intrathecally into 30 day old rats

Lumbar cerebrospinal fluid pressures were assessed immediately following infusion by connecting the intrathecal catheter to a high sensitivity pressure transducer (Model P 23, Statham Corp) Pressures were recorded on a Polygraph (Grass Instrument Co) which was calibrated with a water manometer

Neuro-anatomical observations were made on the distribution of Evans blue in the brains of 5 and 30 day old animals immediately after the 40 and 90 microliter infusions respectively After fixation of the brains in 10 % formalin specimens were dissected and examined with regard to staining of the ventricular system

Sampling and assay of radioactivity in brain

Animals were sacrificed with chloroform and exsanguinated by cardiac removal at 6, 10 15, 30, and 60 minutes from the onset of infusion The brain was then removed and stored at −70° At the time of analysis the frozen brain specimens were warmed to −4°, and dissected into right and left halves Each half brain was homogenized in 10 ml of 0 4 N perchloric acid filtered and reconstituted to a volume of 10 ml The acid extract was then mixed with 10 ml of scintillation solvent (Instagel Packard Instrument Corporation, and counted in a Packard liquid scintillation spectrometer The counting efficiency of this scintillation system was assessed with ^{14}C-toluene and found to be 55 %

Results and Discussion

The albino rat is particularly suited for studying the developing nervous system because the brain is relatively undifferentiated at birth and thus can be studied at a time of maturation, when in other species, the organism is still in the uterus Since, in the rat, the maximal rate of brain growth occurs during the first three weeks of postnatal life, before the period of most rapid body growth, animals of small size weighing from 7 to 75 grams must be used (DAVISON & DOBBING 1968, BASS *et al* 1970) Hence, in an effort to develop a method for the infusion of substances into the brain via the cerebrospinal fluid system during early stages of brain development, 5 and 30 day old albino rats were used These animals weighed 13 8 ± 0 3 grams and 62 ± 2 0 grams, respectively, constituting 7% and 31% of the body weight of adult

50 day old rats In contrast, their respective brain weights were 0 53 ± 0 03 grams and 1 34 ± 0 02 grams, constituting 32% and 77% of adult 50 day old values

In both 5 and 30 day old rats, neuro anatomical observations of the vertebral canal were made to determine the feasibility for catheterization of the spinal subarachnoid space The spinal cord was seen to extend downward to the L-4 vertebral level in 30 day old rats, and occupied the full length of the spinal canal in 5 day old animals However, the diameter of the cord was much smaller than that of the vertebral canal in which it lay Moreover, at the lumbar vertebral level, an extremely wide subarachnoid space exists between the surface of the cord and the bony vertebral wall This relatively large subarachnoid space constituted over 50 % of the total volume of the canals, which had internal transverse diameters of 3 7 mm and 2 5 mm in 30 and 5 day old animals, respectively Hence a catheter of 0 8 mm outer diameter, once placed in the lumbar subarachnoid space, could easily be threaded above the dorsal surface of the cord (fig 1)

After successful intrathecal catheterization of both 5 and 30 day old rats, the total volume of the perfusate and optimal rate of infusion were determined on unanaesthetized animals It has been previously reported that in monkeys and man a complete distribution of drugs throughout the cerebral subarachnoid space and ventricular system could be accomplished by lumbar intrathecal injection of a fluid volume equal to at least one fourth of the estimated total cerebrospinal fluid volume (RIESELBACH *et al* 1962) Based on inulin dilution studies, we have estimated the total spinal fluid volume in the 5 and 30 day old rat to be 120 and 250 microliters respectively (BASS & LUNDBORG unpublished results) Hence, in an effort to deliver substances within an infusion time of 5 minutes, a volume of 40 microliters (33% of total spinal fluid volume) at a rate of 11 2 microliters per minute was used in 5 day old rats

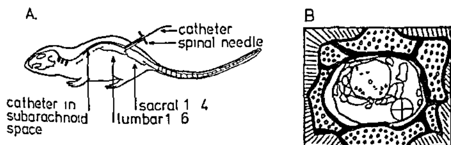


Fig 1 A Anatomical drawing of the rat vertebral column depicting site of penetration of spinal needle and position of catheter B Anatomical drawing of transverse section through vertebral column at L-5 showing the termination of cord cauda equina and relatively large subarachnoid space (+) denotes catheter size and position in the spinal canal drawing magnification ×15

and a corresponding volume of 90 microliters (36 % of total spinal fluid volume) at a rate of 20 4 microliters per minute in 30 day old animals Forty and ninety microliters of a 1 % solution of Evans blue was then infused at the above stated rates into 5 and 30 day old animals, respectively, in an effort to visualize the intracranial distribution of substances delivered by this route All the animals were sacrificed immediately at the end of the infusion 5 minutes after onset In both groups of animals, the dye was seen to be distributed throughout the cerebrospinal fluid system The cisterna magna, basal cisterns, and subarachnoid space above the hemispheres, were completely filled with dye-stained fluid Moreover, coronal sections of the brains showed that both lateral ventricles, the aqueduct, and the 3rd and 4th ventricles were filled with dye (fig 2 & 3)

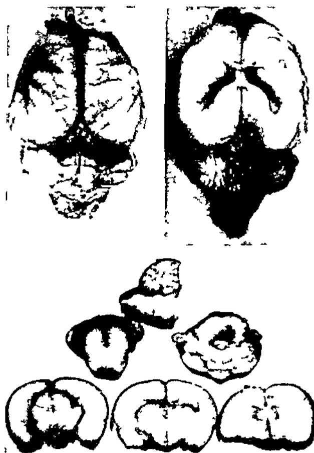


Fig 2 30 day old rat brain immediately following the intrathecal infusion of 90 μl of a 1 % solution of Evans blue (infusion rate 20 4 μl/min) Note black staining of brain surface and complete penetration of the dye into the ventricular system

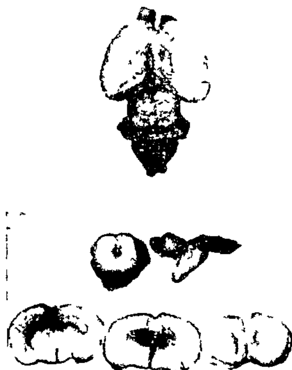


Fig 3 5 day old rat brain immediately following the intrathecal infusion of 40 µl of a 1 % solution of Evans blue (infusion rate 11 2 µl/min) Note black staining of brain surface and complete penetration of the dye into the ventricular system

Since the possibility that a high pressure perfusion artifact sustained during the course of the experiment might adversely effect the interpretation of studies concerned with the metabolism and distribution of drugs, spinal fluid pressure was continuously monitored for 1 hour following infusion (fig 4) A normal spinal fluid pressure of 38 ± 4 mm of water and 18 ± 2 mm of water was found in 30 and 5 day old animals, respectively Evident venous pulsations were seen in pressure tracings from 30 day old rats were not visible in 5 day old animals In 30 day old rats, bilateral jugular vein compression (QUECKENSTEDT s maneuver) produced a six fold increase in pressure, returning to resting levels in 3 minutes A similar pressure rise which returned to normal in 5 minutes could be produced in 5 day old rats by bilateral skull compression At the infusion volumes and rates used for both groups of animals in the present study only a three fold rise in resting spinal fluid pressure was found seven minutes after the onset of infusion, returning to normal resting levels within the 10–15 minutes

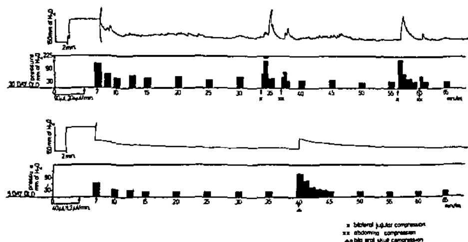


Fig 4 Spinal fluid pressure following intrathecal infusion in 5 and 30 day old rats (µl, µl/min) denotes the volume of fluid delivered in addition to the rate and duration of the infusion Representative polygraph tracings for animals at each age are depicted above the bar graphs showing mean spinal fluid pressures as a function of time (minutes) following infusion Note the increase in spinal fluid pressure following bilateral jugular vein (×) and skull (▲) compression and the relative lack of response to abdominal compression (× ×)

In order to quantify the distribution of a substance in brain after intrathecal infusion, carboxyl-^{14}C inulin was used This substance of large molecular size is not metabolized, shows restricted entry into cells, and is cleared relatively slowly from the cerebrospinal fluid system by the mechanism of bulk flow (HEISEY *et al* 1962, OLENDORF & DAVSON 1967) Hence, the symmetrical distribution of radioactivity in both right and left halves of the brain could be evaluated at varying times following infusion and at different stages of maturation In the 5 day old rat, equilibrium between the right and left halves of the brain was relatively delayed and not achieved until 30 minutes after onset of infusion In contrast, symmetrical distribution of the isotope occurred almost immediately after infusion in 30 day old rats (table 1)

Previous studies have attempted to assess the pharmacological effects of substances on the central nervous system of adult rats by developing techniques for direct injection into the cerebral ventricles (HAYDEN *et al* 1966, NOBLE *et al* 1967, GOODRICH *et al* 1969) Although the lateral ventricle is relatively large in mature rats, it becomes inaccessibly small during early stages of postnatal brain development Furthermore, until recently, cerebroventricular injection techniques necessitated the use of slow, traumatic stereotaxic procedures in association with anaesthesia or immobilizing agents (deBALBIAN VERSTER *et al* 1971) Unfortunately, such drugs have been shown

Table I

Distribution of radioactivity between right and left sides of the brain following intrathecal infusion of carboxyl ^{14}C inulin

Time (minutes)	Amount of radioactivity (in nanocuries)							
	5 day old*				30 day old**			
	N	Right half brain	Left half brain	Right/left brain ratio	N	Right half brain	Left half brain	Right/left brain ratio
6	4	0.86 ± 0.09	1.60 ± 0.14	0.54	6	17.44 ± 1.05	18.21 ± 1.09	0.96
10	4	2.49 ± 0.39	5.72 ± 0.51	0.44	6	10.50 ± 0.53	12.03 ± 0.60	0.87
15	4	4.47 + 0.52	7.57 ± 0.47	0.59	6	6.02 ± 0.48	5.96 ± 0.48	1.01
30	4	11.38 + 0.73	12.24 ± 0.69	0.93	6	0.59 ± 0.01	0.66 ± 0.02	0.89
60	4	16.27 ± 1.02	16.74 + 1.04	0.97	6	0.40 ± 0.01	0.44 ± 0.02	0.91

Values are expressed as nanocuries of ^{14}C inulin per half brain ± S E M   N—number of animals sampled

* Intrathecal infusion of 191.0 nanocuries of ^{14}C inulin in 40 microliter volume at a rate of 11.2 μl/minute

** Intrathecal infusion of 385.3 nanocuries of ^{14}C inulin in 90 microliter volume at a rate of 20.4 μl/minute

to influence profoundly the metabolism and distribution of substances in the brain (SCHANBERG *et al* 1967, LORENZO *et al* 1968) In addition studies have shown that the injection of dyes, radiopaque compounds, and radioactively labelled substances into one lateral ventricle produce (1) an as symetrical distribution between the right and left halves of the brain over a period of 8 hours (ALBANUS *et al* 1969), (2) a diffusion gradient within the cerebrum (RALL *et al* 1962), and (3) a poor cerebrospinal fluid circulation resulting in almost negligible concentrations of the substance in the subarachnoid space above the hemispheres (ALBANUS *et al* 1969) Hence it may be concluded that such techniques do not achieve a complete or symmetrical distribution in either the brain or the cerebrospinal fluid system

In conclusion, the many advantages of the method described may be summarized as follows (1) The catheterization procedure employs simple percutaneous puncture which can be performed on a single animal in ten minutes by a competent technician This allows the investigator to use large numbers of animals in an experiment (2) Infusion into the subarachnoid space can be performed on a waking animal and there is no need to use anaesthetics or immobilizing agents (3) No mortality results from the catheterization or infusion procedure (4) Substances such as Evans blue and carboxyl-^{14}C inulin which have poor ability to pass into the brain when administered via the usual parenteral routes can readily be dispersed throughout the cerebrospinal fluid system and penetrate symmetrically into the extracellular space of both sides of the brain

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REFERENCES

Albanus L., S M Aquilonius A Sundwall & B Winbladh The fate of intracerebroventricular injection of atropine and methylatropine in relation to their pharmacological effects *Acta pharmacol et toxicol* 1969 27 81–96

Ames A M Sakanoue & S Endo Na K Ca Mg Cl concentrations in choroid plexus fluid and cisternal fluid compared with plasma ultrafiltrate *J Neurophysiol* 1964 27 672–681

Bass, N H, M G Netsky & E Young Effect of neonatal malnutrition on developing cerebrum *Arch Neurol* 1970, 23, 289–302

Bito, L Z & H Davson Local variations in cerebrospinal fluid composition and its relationship to the composition of the extracellular fluid of the cortex *Exp Neurol* 1966, 14 264–280

Brightman, M V The intracerebral movement of proteins injected into blood and cerebrospinal fluid of mice *Progr Brain Res* 1968, 29, 19–36

Caley, D W & D S Maxwell Development of blood vessels and extracellular spaces during postnatal maturation of rat cerebral cortex *J comp Neurol* 1970 138, 31–48

Davison, A N & D J Dobbing The developing brain In *Applied Neurochemistry* Eds A N Davison & J Dobbing Philadelphia, F A Davis Co 1968, pp 253–286

de Balbian Verster, F, C A Robinson, C A Hengeveld & E S Bush Freehand cerebroventricular injection technique for unanesthetized rats *Life Sci* 1971, 10, 1395–1402.

Goodrich, C A B, T B Greebey, T B Miller & J R Pappenheimer Cerebral ventricular infusions in unrestrained rats *J appl Physiol* 1969 26, 137–140

Hayden, J F, L R Johnson & R P Maickel Construction and implantation of a permanent cannula for making injections into the lateral ventricle of the rat brain *Life Sci* 1966, 5, 1509–1515

Heisey, S R, D Held & J R Pappenheimer Bulk flow and diffusion in the cerebrospinal fluid system of the goat *Amer J Physiol* 1962, 203 775–781

Lorenzo, A V, J P Hammerstad & R W P Cutler The effect of anesthetic agents on the cerebrospinal fluid clearance of S^{35} sulphate and I^{125} iodide *Biochem Pharmacol* 1968, 17, 1279–1283

Noble, E P, R J Wurtman & J Axelrod A simple and rapid method for injecting ^{3}H norepinephrine into the lateral ventricle of the rat brain *Life Sci* 1967, 6, 281–291

Olendorf W H & H Davson Brain extracellular space and the sink action of the cerebrospinal fluid *Arch Neurol* 1967, 17, 196–205

Rall D P Transport through the ependymal linings *Progr Brain Res* 1968, 29, 159–167

Rall, D P W W Opelt & C S Patlak Extracellular space of the brain as determined by diffusion of inulin from the ventricular system *Life Sci* 1962, 2, 43–48

Rieselbach R E, G DiChiro, E J Freireich & D A Rall Subarachnoid distribution of drugs after lumbar injection *New Engl J Med* 1962, 267, 1273–1278

Schanberg S M, J J Schildkraut & I J Kopin The effects of pentobarbital on the fate of intracisternally administered norepinephrine-^{3}H *J Pharmacol exp Therap* 1967, 157, 311–317

Acta pharmacol et toxicol 1973, 32, 304-313

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Biological and Pharmacokinetic Evidence for Generic Equivalence of Three Imipramine Preparations: Comparison with a New Imipramine Analogue

By

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Abstract The biological availability of three imipramine preparations and an imipramine analogue Leo 640 has been studied using a recently developed biological procedure The isolated rat iris, was used to elucidate the inhibitory power of these drugs on noradrenaline uptake into adrenergic nerve terminals. Irides incubated in patients plasma before and during drug treatment were compared in two series of cross-over stud es on hospitalized patients It was found that the drugs compared seemed to be identical with regard to their effect on noradrenaline neurons It has been hypothesized that the antidepressive effect of this type of tricyclic drugs is brought about by their inhibition of re uptake of monoamines released into central synapses. This method provides a new way for comparing these drugs and their active metabolites at the presumed site of action

Key words Generic equivalence – noradrenaline neurons – tricyclic anti depressants – imipramine

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In recent years it has been frequently pointed out that generic equivalence, ought to imply besides chemical equivalence, availability equivalence and therapeutic equivalence (LEVY 1964, VARLEY 1968, FRIEND 1968, WAHLQUIST 1969) Several reports have appeared on the so-called generic in equivalence of synonymous drugs i e drugs which are chemically equivalent but vary in their dissolution rates and/or absorption rates and consequently are quite different in their bio availability Different absorption characteristics have been shown for generically equivalent commercial preparations of drugs such as chloramphenicol (BARTELLONI *et al* 1969, GLAZKO *et al* 1968, AGUIAR *et al* 1968), phenobarbitone (phenemalum NFN), phenacetin acetylsalicylic acid, sulphadiazine (FINHOLT *et al* 1966) and griseofulvin

(SYMCHOWICZ & KATCHEN 1968) Bio-availability is usually assessed from plasma concentrations of the parent compound Such studies have been published for acetylsalisylic acid (LEVY 1964), phenylbutazone (SEARL & PERNAROWSKI 1967), oxytetracycline (BRICE & HAMMER 1969), isoniazid (GELBER *et al* 1969), and thioridazine (HIRST & KAYE 1971) Fewer studies have been published on the therapeutic equivalence of drugs at the assumed site of action VARLEY (1968) carried out experiments with tolbutamide and blood sugar levels and LOZINSKI (1960) related changes in prothrombin times to pharmaceutical changes of chemically equivalent dicoumarol tablets

In a series of experiments we have studied the biological availability of three imipramine preparations and Leo 640 (ERIKSOO & ROTHE 1970), an imipramine analogue, by measuring the plasma level of the main desmethylated metabolite of imipramine and by utilizing a recently developed biological procedure (BORGÅ *et al* 1970, SIWERS *et al* 1970) This is based on the fact that tricyclic antidepressants inhibit the neuronal uptake of noradrenaline in the adrenergic ground plexus of the rat iris (MALMFORS 1965) This tissue is incubated in the patient s plasma which contains the antidepressant (and its active metabolites)

The prime purpose of the first part of this study was to compare the biological availability of two different pharmaceutical formulations of imipramine, imipramine Geigy (tofranil®) and imipramine ACO, while in the second part imipramine Leo, imipramine-Geigy and Leo 640 were compared Our intention was also to find a dosage of Leo 640 which gave approximately the same biological effect as the imipramine standard

Material and Methods

Patients and drugs

The patients had all been admitted to the psychiatric departments of two general hospitals because of depressive illness of various types and antidepressants were deemed necessary by the attending psychiatrist All the patients were free from drugs for at least 10 days before therapy was initiated Other drugs were not given the only exceptions allowed were occasional doses of anxiolytics – such as diazepam or meprobamate as well as night sedatives (barbiturates) Studies in progress indicate that the metabolism of tricyclic antidepressant drugs is not readily induced by barbiturates However, it has been shown that long term treatment with barbiturates tend to lower the steady state plasma concentration of e g nortriptyline in man

The following imipramine preparations were used imipramine Geigy (tofranil®) as commercial tablets containing imipramine chloride with a crystal size of less than 50 μ imipramine ACO as commercial sugarcoated pills containing imipramine chloride with a crystal size of less than 100 μ and imipramine Leo, as tablets containing imipramine chloride with a crystal size of less than 50 μ

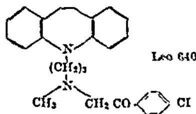
Drug regimen

a) *Cross over with two Imipramine preparations*

The first group included 7 patients who were randomly given either impramine Geigy (tofranil®) or imipramine ACO for at least 10 days Blood samples were drawn on days 9 and 10 and the patients were then switched over to the other drug for 10 days and blood samples were again taken The dose was 25 mg t.i.d

b) *Cross-over with two Imipramine preparations and Leo 640*

The second series of 8 patients received imipramine Leo 50 mg t.i.d., Leo 640 75 mg t.i.d and imipramine-Geigy 50 mg t.i.d The initial drug was chosen at random. As in the first group of patients, each drug was given for at least 10 days before blood samples were taken after which the patients were switched over to the subsequent treatment Leo 640 is an imipramine analogue with one hydrogen in one of the N methyl groups substituted for a p-chlorobenzoyl group



Plasma samples

Blood samples were taken approximately one hour before the mid-day dose They were heparinized and centrifuged immediately the plasma was pipetted off and subsequently frozen and stored at −20° until analysis The plasma samples were kept for various periods of time (up to 6 weeks) before being further processed.

Uptake of ³H noradrenaline in rat iris

Irides of female albino rats (Sprague-Dawley body weight about 200 g) were used. The animals were killed under ether anaesthesia by exsanguination The eyes were then removed and the irides (with the ciliary body attached) were carefully dissected out under a microscope according to MALMFORS (1965) The irides were thereafter preincubated in 1 ml plasma for 15 min at 37° Tritiated noradrenaline HCl (³H NA) (5–10 ci/mmol in a final concentration of 10^{-7} M or 17 ng/ml) was added and the incubation continued for 30 min (JONSSON *et al* 1969) Incubation took place in an atmosphere of 93.5 % O_2 and 6.5 % CO_2 The pH in the plasma was found to be about 7.4 All irides were then rapidly rinsed in buffer solution and incubated for a further 10 min at 37° in Krebs Ringer bicarbonate buffer without amine in order to remove any extraneuronal NA Finally the tissue was solubilized in 0.5 ml soluene® and after the addition of 10 ml toluene scintillation solution the radioactivity was measured in a liquid scintillation spectrometer Non radioactive irides gave blank values

The results are given as % of the NA uptake in irides incubated in plasma drawn immediately before treatment

Measurement of desipramine in plasma

Desipramine the main metabolite of imipramine was measured according to HAMMER & BRODIE (1967)

Dissolution rate studies

The three forms of imipramine were studied according to a USP method (US Pharmacopeia XVIII pp 934-5) a modification of the procedure as described by LEVY & HAYES (1960) Each tablet was weighed and then placed in a small cylindrical stainless steel basket which was immersed in the dissolution medium and rotated at a constant rate of 50 r p m A water bath maintained the temperature at 37 ± 1° The dissolution medium consisted of 800 ml 0 1 N HCl and aliquots of 10 ml were withdrawn for analysis at various time intervals After each withdrawal 10 ml of 0 1 N HCl were added This was taken into account in the calculation

Table 1.

Biological availability of imipramine ACO (I ACO) and imipramine Geigy (I Geigy)

Patient	Drug	Plasma conc of DMI[1] (ng/ml)	Uptake of ^{3}H NA (% of control)
1	I Geigy	7	57
	I ACO	11	51
2	I Geigy	14	58
	I ACO	18	53
3	I Geigy	13	45
	I ACO	20	45
4	I Geigy	27	51
	I ACO	22	55
5	I Geigy	88	31
	I ACO	83	29
6	I Geigy	96	25
	I ACO	76	27
7	I Geigy	117	32
	I ACO	95	33

[1] DMI = desipramine

Significance of mean intraindividual differences

	d	t	n	p[2]
Plasma conc (DMI) I Geigy I ACO	5 29	1 20	7	n s
Uptake I Geigy – I ACO	0 86	0 62	7	n s

Intercorrelations between some variables

	r	t	n	p[2]
DMI conc I Geigy – NA uptake I Geigy	−0 91	4 91	7	⩽ 0 005
DMI conc I ACO – NA uptake I ACO	−0 92	5 25	7	⩽ 0 005

[2] d = mean difference
r = product moment correlation coefficient
n = number of patients
p = estimated by one tailed test

Table 2

Biological availability of imipramine Geigy (I Geigy) Leo 640 and imipramine Leo (I Leo)

Patient	Drug	Plasma conc of DMI[1]	Uptake of ^{3}H NA (% of control)
1	I Geigy	76	20
	Leo 640		25
	I Leo	82	17
2	I Geigy	59	30
	Leo 640		30
	I Leo	28	32
3	I Geigy	206	16
	Leo 640		22
	I Leo	187	14
4	I Geigy	15	41
	Leo 640		36
	I Leo	24	34
5	I Geigy	211	15
	Leo 640		18
	I Leo	160	15
6	I Geigy	190	13
	Leo 640		24
	I Leo	188	17
7	I Geigy	72	19
	Leo 640		22
	I Leo	52	20
8	I Geigy	49	21
	Leo 640		28
	I Leo	51	29

[1] DMI = desipramine

Analysis of variance

Source of variance	df	Sum of squares	Mean square
Compounds	2	73 0833	36 5417
Patients	7	1067.2083	152.4583
Error	14	137 5834	9 8274
F tests			
Compounds	$F(2\ 14)$ =	3 7183	n s
Patients	$F(7\ 14)$ =	15 5136	$p \leqslant 0\,01$

Intercorrelations between some variables	*r*	*t*	*n*	*p*
DMI conc I Geigy – NA uptake I Geigy	−0 77	2 96	8	$\leqslant 0\,025$
DMI conc I Leo – NA uptake I Leo	−0 84	3 79	8	$\leqslant 0\,005$

r = product moment correlation coefficient
n = number of patients
p = estimated by one tailed test

Assay of tablets

Two 25 mg tablets of each brand were pulverized and dissolved in 50 ml 0 1 N HCl under gentle agitation for 0.5 hr and thereafter 100 ml HCl was added The solution was filtered, the first 25 ml discarded and 5 ml diluted to 100 ml with 0 1 N HCl and assayed spectrophotometrically at 251 mμ for total imipramine

Standard curve

0 05 g imipramine hydrochloride was weighed and then dissolved in 200 ml 0 1 N HCl Aliquots of 3, 5, 8 and 10 ml were diluted to 100 ml with 0 1 N HCl and assayed spectrophotometrically at 251 mμ for total imipramine

Results

Plasma samples from patients treated in a cross-over manner with imipramine ACO and imipramine-Geigy were compared with regard to the concentration of desipramine and uptake of ^{3}H-NA in incubated rat irides (table 1) The two preparations of imipramine were found to be equivalent in both respects

Plasma from patients treated with imipramine-Leo, imipramine-Geigy and Leo 640, in essence, performed similarly as regards NA uptake and the two forms of imipramine gave similar plasma concentrations of desipramine (table 2)

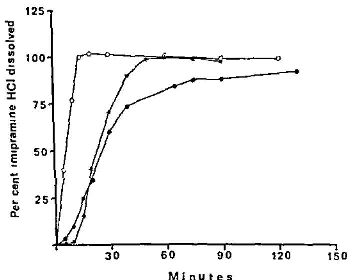


Fig 1 Dissolution rates of three forms of imipramine
● imipramine ACO ○ imipramine Leo and ★ imipramine-Geigy expressed as per cent imipramine HCl dissolved in the medium at different time intervals

Using this method previous experiments have shown a correlation between the plasma concentration of the tricyclic antidepressant nortriptyline and the inhibition of NA uptake (BORGÅ *et al* 1970) Tables 1 and 2 show that there is also a strongly significant negative correlation between the plasma concentration of desipramine and the uptake of ^{3}H NA

The dissolution rates for the three forms of imipramine differed slightly (fig 1), imipramine Leo as a tablet showed the quickest dissolution rate the sugarcoated pill of imipramine ACO being the slowest

Discussion

Documented examples of generic inequivalence concern drugs which have comparatively short half lives such as sulfamethazine (sulfadimidinum NFN) (TARASZKA & DELOR 1969) and chloramphenicol (BELL *et al* 1971) Antidepressant drugs are characterized by a rapid rate of absorption as compared to the rate of elimination (*cf* SJÖQVIST *et al* 1971) Variability in dosage form should therefore not be expected to affect the steady state plasma concentrations of these drugs during continuous treatment Generic equivalence of three commercial preparations of diazepam given in repeated doses has recently been demonstrated and is to be expected since diazepam is rapidly absorbed and slowly metabolized with a half life over 24 hours (BERLIN *et al* 1972)

In controlled clinical trials it is fundamentally important that the reference compound has the same bio availability as the original pharmaceutical product containing the same chemical substance This reference compound often has however to be pharmaceutically reformulated so as not to violate the double blind design of the intended study This is the case with imipramine Geigy (tofranil®) which is labelled with the manufacturer s name and must therefore be altered before initiating a clinical trial

It was therefore of great importance to ascertain if imipramine Leo and imipramine Geigy had the same biological availability before beginning a proposed clinical trial of Leo 640 in which imipramine was to be the reference substance If this were not the case the data obtained in such a trial would not have had any clear relevance to the literature describing the effects of imipramine Geigy (tofranil®)

The data obtained from the dissolution rate studies showed somewhat different rates for the three forms of imipramine studied Nonetheless in the first cross-over study, there was no significant difference between the two commercial forms of imipramine (Geigy and ACO) studied as regards their bio-availability Similarly, in the second cross-over study no significant difference was seen between the two imipramines Geigy *and Leo It may*

thus be safely assumed that these three formulations of imipramine are not only chemically but also biologically equivalent

In the second experiment a dose regimen was chosen for Leo 640 (75 mg t i d) in an attempt to match imipramine (50 mg t i d) in terms of biological availability The doses chosen were approximately equimolar (Imipramine M = 280, Leo 640 M = 455 4) Statistical analysis showed no difference between Leo 640 and the two imipramine preparations as regards the effects of endogenous plasma concentration on the uptake of NA in the rat iris The metabolic fate of Leo 640 is not known but im ipramine cannot be formed from this compound

It has been suggested that the depressive state is associated with a disturbed monoamine balance in the central system (CARLSSON 1965, SCHILDKRAUT & KETY 1967) The present hypothesis is that tricyclic antidepressants potentiate the effect of monoamines released into the central synapses by inhibition of their re uptake It can thus be inferred that the drugs studied here are essentially, identical with regard to their effect on NA neurons On the basis of our experiments a controlled clinical trial of Leo 640 (75 mg t i d) in comparison with imipramine Leo (50 mg t i d) has been initiated The data described unequivocally reveal that under these conditions this new drug and the reference compound will be compared on an equal basis with respect to the effect on NA uptake and also as regards their therapeutic effect on depressive states to the extent by which they may be relieved by NA uptake inhibition

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REFERENCES

Aguiar A J L M Wheeler S Fussari & J E Zelmar Evaluation of physical and pharmaceut cal factors involved in drug release and availability from chlor amphenicol capsules *J Pharm Sci* 1968 11 1844–1850

Bartellon P J F M Calia B H Minchew W R Beisel & H L Ley Jr Absorption and excret on of two chloramphenicol products in humans after oral admin strat on *Amer J Med Sci* 1969 258 203–208

Bell H H Johansen P K Lunde H A Andersgaard P Finholt T Midtvedt E Holum B Martinussen & E D Aarnes Absorption and dissolut on char acteristics of 14 different oral chloramphen col preparations tested on human male subjects *Pharmacol* 1971 5 108–120

Berlin, A, B Siwers, S Agurell, A Hiort, F. Sjöqvist & S Ström Determination of bioavailability of diazepam from steady state plasma concentration data *Clin Pharmacol Therap* 1972, **13**, 733–744

Borgå, O, B Hamberger, T Malmfors & F. Sjoqvist The role of plasma protein binding in the inhibitory effect of nortriptyline on the neuronal uptake of epinephrine *Clin Pharmacol Therap* 1970, **11**, 581–588

Brice, G W & H F. Hammer Therapeutic nonequivalence of oxytetracycline capsules. *JAMA* 1969, **208**, 1189–1190

Carlsson, A Drugs which block the storage of 5 hydroxytryptamine and related amines In *Handbuch der Experimentellen Pharmakologie* Ed · V Erspamer Springer Verlag, Berlin Göttingen Heidelberg, 1965, pp 529–592

Eriksoo, E & O Rothe Chemistry and pharmacology of a new potential anti depressant *Arzneim-Forsch (Drug Res)* 1970, **20**, 1561–1569

Finholt, P, H Kristiansen, O C. Schmidt & K Wold Effect of different factors on dissolution rate of drugs from powders, granules and tablets *I Meddelelser fra norsk farmaceutisk selskap* 1966, **28**, 17–30

Friend D G Generic drugs and therapeutic equivalence *JAMA* 1968, **206**, 1785

Gelber, R., J Jacobsen & L. Levy A study of the availability of six commercial formulations of isoniazid *Clin Pharmacol Therap* 1969, **10**, 841–848

Glazko, A J, A W Kinkel, W C Alegnani & E. L. Holmes An evaluation of the absorption characteristics of different chloramphenicol preparations in normal human subjects *Clin Pharmacol Therap* 1968, **9**, 472–483

Hammer, W & B B Brodie Application of isotope derivative technique to assay of secondary amines Estimation of desipramine by acetylation of H³-acetic anhydride *J Pharmacol Exp Therap* 1967, **157**, 503–508

Hirst, C A & R C Kaye On the effect of pharmaceutical formulation on thioridazine absorption *J Pharm Pharmacol* 1971, **23**, Suppl, 247 S

Jonsson, G, B Hamberger, T Malmfors & C Sachs Uptake and accumulation of ³H noradrenaline in adrenergic nerves of rat iris Effect of reserpine, monoamine oxidase and tyrosine hydroxylase inhibition *Eur J Pharm* 1969, **8**, 58–72.

Levy, G & B A Hayes Physiochemical basis of the buffered acetylsalicylic acid controversy *New Eng J Med* 1960, **21** 1053–1058

Levy, G Effect of dosage form on drug absorption a frequent variable in clinical pharmacology *Arch Int Pharmacodyn* 1964 **152**, 59–68

Lozinski E Physiological availability of dicumarol *Canad med Ass J* 1960 **83** 177–178

Malmfors, T Studies of adrenergic nerves The use of rat and mouse iris for direct observations on their physiology and pharmacology at cellular and subcellular levels *Acta Physiol Scand* 1965, **248**, Suppl 1–64

Schildkraut, J & S Kety Biogenic amines and emotion *Sci* 1967, **156** 21–30

Searl, R O & M Pernarowski The biopharmaceutical properties of solid dosage forms I An evaluation of 23 brands of phenylbutazone tablets *Canad med Ass J* 1967, **96**, 1513–1520

Siwers, B, U Freyschuss B Hamberger D Tuck, T Malmfors & F Sjöqvist A quantitative approach to the initial clinical trial of tricyclic antidepressants A comparison of Leo 640 and nortriptyline *Eur J Clin Pharmacol* 1970 **3** 12–17

Sjöqvist, F, B Alexanderson, M Asberg L. Bertilsson, O Borgå, B Hamberger & D Tuck Pharmokinetics and biological effects of nortriptyline in man *Acta pharmacol et toxicol* 1971, **29**, suppl 3, 255–280

Symshowics, S & B Katchen Griseofulvin absorption in man after single and repeated treatments and its correlation with dissolution rates *J Pharm Sci* 1968, 57, 1383–1386

Taraszka, M J & A Delor Effect of dissolution rate of sulfamethazine from tablets on absorption and excretion of sulfamethazine *J Pharm Sci* 1969, 58, 207–210

Wahlqvist, S Har synonympreparat samma effekt? *Lakartidningen* 1969, 66, Supl II, 50–56

Varley, A B The generic inequivalence of drugs *JAMA* 1968, 206, 1745–1748

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Bile Duct Proliferation in Pigs Fed the Food Colour Orange RN

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Key words Bile duct – pigs – food colour – orange RN

The azo-dye Orange RN, (CI No 15970), 1-phenylazo 2 naphthol-6-sulphonic acid, is used for beverages and food e g. sausages Oral administration to rats and mice may produce methaemoglobinaemia, anaemia reticulocytosis, increase in the weight of the spleen with increased content of iron and marked production of Heinz bodies (DACRE 1969, GAUNT *et al* 1971)

The investigations of Orange RN were made on 24 SPF pigs of Danish Landrace The pigs were divided into four groups of 3 females and 3 males each to which the dye was fed mixed with the diet in doses of 0 10, 40 and 160 mg/kg body weight/day The experiment lasted for three months and included observations of a number of clinical haematological, and pathological anatomical parameters

Results and Discussion

Dosing with Orange RN produced changes in the blood picture similar to those found in small rodents Clinically the pigs showed transient symptoms of paleness In addition changes in the colour and consistency of the faeces were observed

A macroscopic examination of the liver showed in a few of the animals of the middle dosage group a diffuse slight fibrosis of the liver The pigs from the highest dosage group, however had a severe interstitial fibrosis and multiple nodular hyperplasias of the parenchyma

Histological examination revealed a slight increase in the interstitial connective tissue in the liver in a few pigs in the control and the lowest dosage group, whereas this was a constant finding in the other groups In agreement with the macroscopic finding an extensive formation of fibrous connective tissue was demonstrated in the highest dosage group along the triads and the interlobular septa as well as pseudolobulation of liver acini



Fig 1 Normal liver tissue with the interlobular septum in which a bile duct is seen (arrow) 0 mg/kg body weight Haematoxylin and eosin. Magnification ×400



Fig 2 Along the interlobular septum a small area with proliferation of bile ductule epithelium cells is seen (arrow) 0 mg/kg body weight Haematoxylin and eosin Magnification ×400



Fig 3 A proliferating bile ductule (arrows) stretching in from the interlobular septum between the rows of liver cells 40 mg/kg body weight Haematoxylin and eosin Magnification ×400

the incubation was continued for 60 min The incubates were spun down at 120,000×g_{max} for 30 min The radioactivity content of the medium and of the washed pellet (after digestion with Soluene) was measured After quench correction and measurement of protein (LOWRY *et al* 1951) the binding of label was calculated as d p m per mg protein

The SPM fraction was incubated with DHM-^{3}H in the presence or absence of methadone antipodes It was found (table 1, expts. 1–4) that levomethadone was an efficient inhibitor of DHM-binding while dextromethadone was inactive or at least markedly less active Since only levomethadone is an active narcotic analgesic (MELLETT & WOODS 1963) this indicates the functional significance of the binding In similar experiments a number of other narcotic analgesics and the narcotic antagonists, nalorphine and naloxone at 10^{-8} M concentration also inhibit the binding (not shown) It was also observed that pretreatment with saline for 4 days (expt 5), of morphine for 4 days (expt 6) or for 17 days (expt 7) did not markedly change the total or stereospecific binding (table 1)

Earlier reports on the uptake of narcotic analgesics by subcellular fractions *in vitro* give conflicting results VAN PRAAG & SIMON (1966) used a rat total brain homogenate and found no evidence for selective uptake NAVON & LAJTHA (1970) found an accumulation of 10^{-5} or 10^{-3} M morphine-^{3}H in the mitochondria but not in the nuclei, nerve ending particles or myelin. Uptake in mitochondria as well as in nuclei (no other fraction studied) was

Table 1

Effects of non labelled levomethadone or dextromethadone (10^{-8} M) on the binding of DHM-^{3}H to the SPM fraction Ten animals were used in each experiment. Groups 6 and 7 received daily intraperitoneal injections of morphine in linearly increasing doses (expt 6 from 20 to 35 mg/kg on day 4 expt 7 from 20 to 100 mg/kg on day 17) Group 5 received only vehicle (saline) for 4 days The experiments were run in duplicate

Expt No	Addition I None		Addition II Levomethadone		Addition III Dextromethadone	
1	2911	3253	1625	1634	2760	2881
2	3352	3570	1672	1699	2771	2938
3	2467	2753	1695	1795	2538	3068
4	3070	3150	1600	1820	2180	2892
	3067 ± 124*		1694 ± 28*		2754 ± 99*	
5	2212	2848	1635	1712	2896	2960
6	2654	2885	1567	2012	2879	3021
7	2646	2736	1266	1326	1843	2306

*Means ± S E M expts 1–4 P difference I II < 0.001, P difference I III > 0.05

inhibited by nalorphine (10^{-5} or 10^{-3} M) HUG & OKA (1971) found a comparatively high uptake of DHM (10^{-7} M) in myelin, cholinergic and noncholinergic SPM fractions However, 10^{-4} M nalorphine did not affect the uptake while 10^{-3} M nalorphine reduced uptake by 14–24 % in all the fractions studied (including nuclei, mitochondria, microsomes, thus revealing no specificity) GOLDSTEIN *et al* (1971) with labelled levorphanol at 4×10^{-5} M and the corresponding non labelled optical antipodes at 4×10^{-3} M, found evidence for a stereospecific uptake in the nuclear fraction, particularly in the nuclear membranes, but very little in crude mitochondrial or crude microsomal fractions However, the stereospecific uptake of the nuclear fraction was only about 2 % of the total amount (very considerable uptake)

In experiments of the present type it is essential for their interpretation to analyse the specificity of the uptake In the present work this was done by comparing the affinities of the synthetic, to the opiates structurally unrelated, optical antipodes of methadone of which only one is a narcotic analgesic Such an analysis is lacking in several of the earlier reports

The earlier investigators used labelled narcotics at a considerably higher concentration The specific binding demonstrated in the present report is almost saturated at 10^{-8} M of DHM (cf table 1) and levorphanol and nalorphine have a comparable affinity (unpublished)

Consequently for technical reasons the earlier investigators would not observe the present high affinity system The high affinity binding material could well be the actual narcotic receptor

Acknowledgements

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REFERENCES

Goldstein A L I Lowney & B K Pal Stereospecific and nonspecific interactions of the morphine congener levorphanol in subcellular fractions of mouse brain *Proc Nat Acad Sci USA* 1971 68 1742–1747

Gray E G & V P Whittaker The isolation of nerve endings from brain an electron microscopic study of cell fragments derived by homogen zat on and centrifugation *J Anat (London)* 1962 96 79–88

Hug Jr C C & T Oka Uptake of dihydromorphine-3H by synaptosomes *Life Sciences* 1971 10 201–213

Lowry O H N J Rosebrough A L Farr & R J Randall Protein measurement with the Folin phenol reagent *J Biol Chem* 1951 193 265 275

Mellett L B & L A Woods Analgesia and add ction *Fortschr Arzneimittelforsch* 1963 5, 154–267

Navon, S & A Lajtha Uptake of morphine in particulate fractions from rat brain. *Brain Res* 1970, 24 534–536

Terenius L. Specific uptake of narcotic analgesics by subcellular fractions of the guinea pig ileum *Acta pharmacol et toxicol* 1972, 31 Suppl I 50

van Praag D & F J Simon Studies on the intracellular distribution and tissue binding of dihydromorphine 7 8 H³ in the rat *Proc Soc Exp Biol Med* 1966 122 6–11

Whittaker, V P I A Michaelson & R J A Kirkland The separation of synaptic vesicles from disrupted nerve-ending particles ("synaptosomes") *Biochem J* 1964 80 293–303

Acta pharmacol et toxicol 1973, 32, 321–336

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Cyclic AMP as a Mediator of the Relaxing Action of Papaverine, Nitroglycerine, Diazoxide and Hydralazine in Intestinal and Vascular Smooth Muscle

By

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Abstract Papaverine and nitroglycerine relaxed rabbit colon muscle The relaxation was preceded by an increase of the cyclic AMP content of the muscle There was a correlation between the degree of relaxation and the increase of cyclic AMP A reduction of ATP content was obtained with these drugs this effect was not present in the Ca^{+} poor preparation in spite of the fact that the cyclic AMP level was still increased Papaverine but not nitroglycerine reduced the phosphodiesterase activity of the intact rabbit colon In a homogenate of colon muscle a marked inhibition of phosphodiesterase activity was obtained The phosphodiesterase activity from different cell fractions was also studied Phosphodiesterase from the cytoplasmic fraction was activated by papaverine and nitroglycerine, while that of a Ca^{++} binding microsomal fraction like that of mitochondria was inhibited Diazoxide and hydralazine relaxed bovine mesenteric artery but not rabbit colon. The relaxation was associated and correlated with an increase in the cyclic AMP content A reduction in the ATP content and an activation of phosphorylase *a* activity was also obtained the ATP reduction was Ca^{++} dependent A weak inhibitory effect on phosphodiesterase activity was observed in homogenate of the artery after diazoxide Hydralazine activated phosphodiesterase in a homogenate of mesenteric artery in a low concentration but inhibited it in a high concentration It is suggested that cyclic AMP mediates at least partly, the relaxing action of these four drugs probably by stimulating a Ca^{++} binding process in the smooth muscle cell

Key words Cyclic AMP – mediator – rabbit – smooth muscle – papaverine – nitroglycerine – diazoxide – hydralazine

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In smooth muscle cyclic AMP is of significance in the regulation of the tension and carbohydrate metabolism Stimulation of adrenergic β receptors in rabbit colon muscle and bovine mesenteric artery is associated with an increase in the level of cyclic AMP The relaxing action and metabolic

effects – phosphorylase *a* activation and reduction of the ATP content – mediated by β-receptor stimulation are preceeded by and also quantitative correlated to an increase in the cyclic AMP level (ANDERSSON & MOHME LUNDHOLM 1970, ANDERSSON 1972a, 1973a & b)

The contraction relaxation cycle in various kinds of muscle is probably regulated by Ca^{++} The sarcoplasmic reticulum plays an important role in skeletal muscle by regulating the free myoplasmic Ca^{++}-concentration (WEBER *et al* 1963, EBASHI & ENDO 1968 RÜEGG 1971) Microsomal fractions which in the presence of ATP bound Ca^{++} have been isolated from uterine smooth muscle (CARSTEN 1969) and from rabbit colon muscle (ANDERSSON *et al* 1971) Adrenergic β receptor agonists and exogenously cyclic AMP increase the Ca^{++}-binding capacity of the latter preparation (ANDERSSON & NILSSON 1972) It has been suggested that cyclic AMP regulates the muscle tension by influencing the Ca^{++}-level of the muscle cell (ANDERSSON 1972a & b)

KUKOVETZ & PÖCH (1970) demonstrated a relationship between drugs which relax the coronary vessels and their ability to inhibit the phosphodiesterase (PDE) activity i e the enzyme hydrolyzing cyclic AMP Papaverine is among the most effective PDE inhibitors There is a correlation between the degree of inhibition and the relaxing effect (PÖCH 1971 TRINER *et al* 1971) Papaverine is an effective smooth muscle relaxing agent which non-competitively inhibits the action of most contracting agents (SIMONIS *et al* 1969) Like the catecholamines it has a positive inotropic action (KUKOVETZ & PÖCH 1970)

Diazoxide is an anti hypertensive agent which competitively antagonizes the contracting action of Ba^{++} and Ca^{++} on isolated vessels (WOHL *et al* 1967 & 1968) SENFT *et al* (1968) found that diazoxide like some other thiazide derivatives inhibits the PDE activity of liver and skeletal muscle and stimulates glycogenolysis Glyceryl trinitrate like other organic nitrates and sodium nitrite is an effective smooth muscle relaxing agent which especially influences the tone of the larger arteries and veins KUKOVETZ *et al* (1969) did not observe any effect of sodium nitrite on the PDE activity of coronary vessels LEVY & WILKENFELD (1968) reported however that nitroglycerine potentiates the inhibitory response of rat uterus to nor adrenaline and that the action of the nitrate itself is potentiated by theophylline The latter observation might indicate that organic nitrates influenced cyclic AMP metabolism in smooth muscle A positive inotropic action of nitroglycerine on human papillary muscles has been described (STRAUER & AVENHAUS 1972) Hydralazine is an effective anti hypertensive agent with a direct action on the vascular smooth muscle (ABLAD 1963)

With regard to the role of cyclic AMP in the mediation of smooth muscle relaxation on stimulation of adrenergic β receptors it was of interest to

study if cyclic AMP had the same function in mediating the relaxing effects of other drugs Preliminary reports covering some of these results have been published (ANDERSSON *et al* 1971, ANDERSSON & NILSSON 1972)

Methods

The experiments were performed on colon muscle from rabbit and bovine mesenteric arteries The muscles were mounted in special holders and the muscle tension was recorded isometrically by a force transducer (FT 03) on a Grass polygraph The dissection and mounting of the preparations have been described previously (ANDERSSON & MOHME LUNDHOLM 1970, ANDERSSON 1973a) The incuba tion medium was a Krebs buffer solution containing 11 5 mM glucose and aerated with 95 % O_2 + 5 % CO_2 at 37° For the preparation of Ca^{++} poor muscles the procedure described by ANDERSSON (1972a & 1973a) was used

The relaxing effect of the substances was studied on the contracted muscle preparation In colon muscle, carbacholine ($1\ 7 \times 10^{-7}$ g/ml) was used to increase the tension The mesenteric arteries were contracted by histamine (1×10^{-6} g/ml) The relaxing agents were added 5–10 min later when the tension had reached a constant level

At the end of each experiments the muscle preparations were quickly frozen in frigen 11, containing solid CO_2 at −80° The cyclic AMP content was determined according to KAKIUCHI & RALL (1968) with a modification described by ANDERSSON (1972b)

The phosphodiesterase activity was assayed according to the method of POCH (1971) The crude enzyme preparation was prepared as follows the muscles were homog enized with the 10 fold volume of a Tris buffer solution (8×10^{-2} M pH 7 0) and centrifuged for 10 min at 2 000 × g (0°) The supernatant obtained after this cen trifugation was used as enzyme preparation The crude enzyme was incubated with a mixture of the following composition Tris HCl (5×10^{-2} M pH 7 5) Mg acetate (4×10^{-3} M) 5 AMP (1×10^{-3} M) and ^{14}C-cyclic 3 5 AMP (1×10^{-7} or 1×10^{-4} M) The reaction volume was 0 5 ml, phosphodiesterase corresponding to 5 mg wet weight of crude extract was used

After incubation at 37° for 30 min the reaction was stopped by the addition of $ZnSO_4$ and the reaction product precipitated quantitatively by Ba $(OH)_2$ The efficience of this precipitation was controlled by chromatography The different adenosine compounds were separated on Avicel cellulose TLC plates or What man paper No 1 with a solvent system consisting of isopropanol/NH_4OH/water (7 1 5 1 5) (GOLDBERG *et al* 1969) or 96 % ethanol/2 M ammonia/2 M acetic acid (100 19 21) (OSNES *et al* 1972) The spots were identified by UV light and the radio active compounds by a Berthold Thin layer scanner II After elution of the spots the activity of the labelled compounds was determined by a liquid scintillation technique By this procedure it was found that 5 AMP was totally precipitated by the $ZnSO_4$ $Ba(OH)_2$ method and no detectable activity of labelled compounds was found in the adenosine region No correction for the PDE values with regard to the effect of the dilution on the degree of inhibition was made in these experiments

In other experiments the drugs were added to the muscle homogenate in fixed concentrations and the PDE activity was determined When the PDE activity of cell fractions was determined, the fractions were prepared according to CARSTEN (1969)

The muscle homogenate was centrifuged for 15 min at 2 500 × g the pellet obtained contained cell debris and nuclei The supernatant was centrifuged at 15 000 × g for 20 min This yielded a pellet consisting of mitochondria and large vesicles After centrifugation of the supernatant at 40 000 × g for 90 min the pellet was re suspended and placed on a sucrose gradient Centrifugation at 45 000 × g for 2 hours revealed three protein layers The fractions were suspended and washed in Tris buffer solution (8 × 10⁻² M) pH 7 0 Papaverine (4 × 10⁻⁴ g/ml) or glyceryl trinitrate was then added to parts of these fractions in the same concentrations which relaxed the intact muscle. Hexosephosphates ATP, CrP and phosphorylase activity were determined as described by Andersson & Mohme Lundholm (1970)

The following drugs were used

Carbaminoyl choline chloride (karbacholin®, ACO)
Diazoxide (hyperstat®, Schering Co)
Glyceryl trinitrate (nitroglycerin®, ACO)
Histamine chloride (histamin®, ACO)
Hydralazine chloride (CIBA)
Papaverine sulphate (papaverin®, ACO)

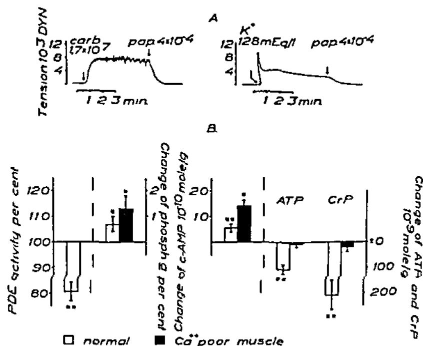


Fig 1A The relaxing effect of papaverine (4 × 10⁻⁴ g/ml) (pap) on colon muscle contracted by carbacholine (1 7 × 10⁻⁷ g/ml) (Carb) and K⁺ high Krebs solution B Changes in the phosphodiesterase and phosphorylase *a* activity and content of cyclic AMP ATP and CrP of colon muscle 60 sec after addition of papaverine (4 × 10⁻⁴ g/ml) Mean ± S E M (n = 7–9) The statistical significance in paired tests from the same muscle is denoted by * = P = < 0 05 ** = P < 0 01, *** = P < 0 001

Table 1.

The effect of papaverine (4×10^{-4} g/ml) on the content of cyclic AMP and the degree of relaxation in rabbit colonic muscle Muscles were frozen 15, 60 and 180 sec after addition of papaverine Mean ± S E M n = 5–6 Significance levels * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$

	cAMP $\times 10^{-10}$ mol/g	Relaxation per cent
Control value	9 7 ± 1 2	-
Δ Papaverine 15 sec	+ 3 0 ± 0 2**	4 9 ± 2 4
Δ Papaverine 60 sec	+ 5 6 ± 1 2**	61 9 ± 9 1**
Δ Papaverine 180 sec.	+ 15 0 ± 2 3**	100 ± 0 0***

Results

Effect of papaverine on the cyclic AMP content and the carbohydrate metabolism and the high energy phosphate compounds of rabbit colon Papaverine (4×10^{-4} g/ml) had a relaxing effect both on the normal rabbit colon muscle and when it was depolarized with 128 meq/l K^+ (fig 1a) The cyclic AMP content in the control muscles was 9 7 ± 1 2 $\times 10^{-10}$ mol/g wet weight This rather high value depends on the fact that carbacholine had increased the basal cyclic AMP level by about 65 % (ANDERSSON 1973c) An increase of the cyclic AMP content was observed 15 sec. after the addition of papaverine, just before the muscle started to relax The increase was still more marked after 60 and 180 sec (table 1) The relaxing effect (y) was correlated with the increase in cyclic AMP content (x) ($r = 0.79$, $P < 0.02$) (fig 2)

To investigate if the changes in cyclic AMP content were associated with the same metabolic effects as stimulation of the adrenergic β-receptors, the phosphorylase *a* activity and the content of high energy phosphate compounds were determined There was a rather moderate increase in the phosphorylase *a* activity after papaverine (fig 1) When the changes in phosphorylase *a* activity (x) were correlated with the relaxing action (y), a negative correlation was however, obtained, ($r = -0.7$, $P < 0.05$ fig 3) A more marked relaxation was thus associated with a smaller increase in the phosphorylase *a* activity than a weaker relaxation

When the ATP and creatine phosphate (CrP) contents were determined

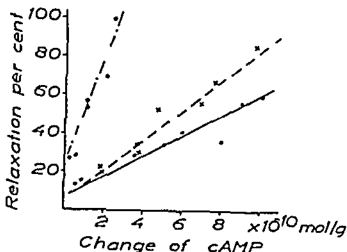


Fig 2. Correlation between the degree of relaxation (100 % total relaxation) and the increase in cyclic AMP content for some drugs in rabbit colon muscle ●——● nitroglycerine ×——× isoprenaline, ●——● papaverine

60 sec. after the addition of papaverine, a marked decrease occurred The ATP-reduction was Ca^{++}-dependent, no decreasing action of ATP or CrP contents was observed in the Ca^{++}-poor muscle, papaverine still increased the cyclic AMP content and activated phosphorylase *a* under these conditions (fig 1) There was a correlation between the degree of relaxation and the change of ATP content of the muscle (fig 3) The coefficient of correlation was -0.90, ($P < 0.02$)

Influence of papaverine on the PDE-activity of whole muscle homogenate and different cell fractions

The mean basal PDE-activity of rabbit colon was 12.0 ± 1.0 nmol of substrate hydrolyzed during 30 min per 5 mg of tissue wet weight There was a reduction in the phosphodiesterase activity of about 20 % in the whole muscle when it was relaxed by papaverine (4×10^{-4} g/ml) (fig 1) The negative correlation between relaxation and the phosphorylase *a* activating effect, which was in contrast to the same relationship after isoprenaline (fig 3) where a positive correlation was observed (ANDERSSON & MOHME-LUNDHOLM 1970), indicated a closer study of how papaverine influenced the PDE-activity of subcellular fractions of smooth muscle

Most of the activity of the enzyme appeared in the cytoplasmic, mitochondrial and the Ca^{++}-binding microsomal fractions Papaverine (4×10^{-4} g/ml) inhibited the PDE-activity in all these fractions except the

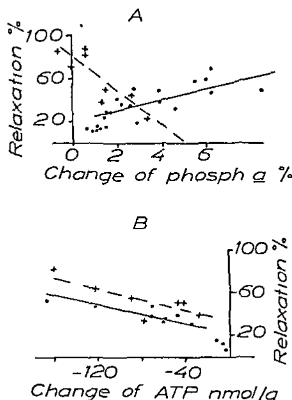


Fig 3 Correlation between A the degree of relaxation and the change in pho phorylase a activity B the degree of relaxation and the decrease of ATP content i rabbit colon after addition of isoprenaline (●——●) and papaverine (x— —x)

cytoplasmic fraction In this fraction papaverine instead significantl activated phosphodiesterase (table 2) The most marked effect of papaverin was its inhibitory action (70 90 %) on the PDE-activity of the microsom fractions, the fraction isolated at a sucrose gradient between 35-45 % bein the most sensitive (table 2)

Relaxing and metabolic effects of nitroglycerine on rabbit colon

Glyceryl trinitrate (2 5 × 10^{-5} g/ml) induced an almost complete relaxa tion of the colon muscle contracted by carbacholine If the muscle wa contracted and depolarized by a K^+-high Krebs solution (128 meq /l) th effect was still present (fig 4A) The relaxation induced by nitroglycerin was preceded (after 15 sec) by an increase in the cyclic AMP content o 0 45 ± 0 1 nmol/g (P < 0 01) from a basal value of 2 0 ± 0 1 nmol/g The cyclic AMP increasing effect

Table 2

Phosphodiesterase activity in different cell fractions from rabbit colonic muscle. The effect of papaverine (4×10^{-4} g/ml) on the phosphodiesterase activity of the different fractions is expressed in per cent of the basal activity in the fraction which is 100 %. Number of tests was 6. The significant level of difference between treated and untreated phosphodiesterase is denoted as in table 1.

Fractions	Basal activity pmol/mg protein/hr	PDE activity in per cent of control activity after papaverine
Nuclei		
cell debris	5.1 ± 0.7	68.6 ± 6.7**
Mitochondria	12.9 ± 3.5	49.6 ± 14.5*
Supernatant	15.8 ± 3.4	138.9 ± 10.8*
Microsomes		
at 35 % sucrose	15.5 ± 3.1	30.0 ± 5.4***
Microsomes		
at 35–45 % sucrose	8.8 ± 2.7	9.1 ± 2.7***
Microsomes		
at 45–55 % sucrose	0.6 ± 0.2	29.8 ± 8.6**

muscle. The increase in cyclic AMP was about 50 per cent of the control value after 60 sec. (fig. 4). The degree of relaxation and increase in cyclic AMP were correlated ($r = 0.76$, $P < 0.05$) (fig. 2). The regression line was, however, much steeper after nitroglycerine than after papaverine and isoprenaline which may indicate a more localized action of the relaxing mechanism by nitroglycerine (fig. 2). Glyceryl nitrate moderately increased the phosphorylase *a* activity and decreased the content of ATP and CrP, the latter effect was dependent on the presence of Ca^{++} (fig. 4 B).

Influence of nitroglycerine on the PDE-activity of whole muscle homogenate and different cell fractions

Nitroglycerine had no significant inhibitory effect on the PDE activity of the muscle *in vitro* (fig. 4 B). In a homogenate of colon muscle, to which nitroglycerine was added, the activity was however markedly influenced, (table 3). In these tests nitroglycerine in concentrations of 1×10^{-5}–5×10^{-4} g/ml very markedly inhibited the phosphodiesterase activity. The effect of nitroglycerine was also studied on the microsomal and cytoplasmic phosphodiesterase activities. The cytoplasmic phosphodiesterase was activated but the microsomal phosphodiesterase was very markedly inhibited. Nitroglycerine at a concentration of 1×10^{-4} g/ml totally inhibited the enzyme activity (table 3).

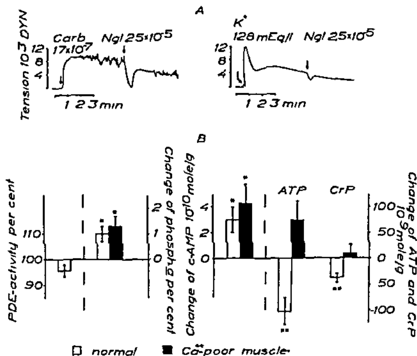


Fig 4A The relaxing effect of nitroglycerine (Ngl) (2.5×10^{-5} g/ml) on colon muscle contracted by carbacholine (Carb) or K^+ high Krebs solution

B Influence of nitroglycerine (2.5×10^{-5} g/ml) on the same parameters as in fig 1 The muscles were frozen 60 sec after the addition of nitroglycerine The control value of cyclic AMP was in normal muscle 1.1 ± 0.3 nmol/g and in Ca^{++} poor muscle 0.8 ± 0.2 nmol/g Mean ± S E M (n=6–9) Statistical significance as in fig 1

Relaxing and metabolic effects of diazoxide and hydralazine on bovine mesenteric artery

Diazoxide (1.5×10^{-4} g/ml) and hydralazine (3×10^{-6} g/ml) did not induce any relaxation of rabbit colon muscle Both substances however induced a relaxation of bovine mesenteric artery Diazoxide was the most potent of the two agents, and the relaxation appeared after a shorter time than after hydralazine (fig 5 A and 6) The tissue level of cyclic AMP was increased by about 40 % ($+ 2.3 \pm 0.6 \times 10^{-10}$ mol/g) from a control value of $5.6 \pm 0.5 \times 10^{-10}$ mol/g 30 sec after the addition of diazoxide After 3 min the increase in cyclic AMP was more pronounced (fig 5 D) Relaxation and increase in cyclic AMP were correlated to about the same degree as after isoprenaline (fig 5 B) Diazoxide stimulated the carbohydrate metabolism to about the same extent as isoprenaline, thus the phosphorylase *a* activity and the content of hexosephosphates were increased (fig 5 C) The ATP and CrP contents of the muscle were reduced after diazoxide, this

Table 3

The effects of nitroglycerine and diazoxide hydralazine on the phosphodiesterase activity of rabbit colon homogenate and fractions, and bovine mesenteric artery homogenate Nitroglycerine and diazoxide were tested at a substrate concentration of 10^{-4} M but hydralazine at a concentration of 10^{-7} M Mean ± S E M (n=6) + = activation, − = inhibition The values are expressed as % of control activity

Conc of Drug	Nitroglycerine			Diazoxide	Hydralazine
	Colon homogenate	40000 × g supernatant	35–45 % microsomal fraction	arterial homogenate	arterial homogenate
10^{-5}	− 58 4 ± 3 4***	+ 24 8 ± 1 8***	− 75 4 ± 12.3***	−	+ 6 3 ± 3 0
10^{-4}	− 62 1 ± 3 1***	+ 22 8 ± 3 0**	− 100 0 ± 14 8**	− 4 7 ± 1 3*	+ 13 0 ± 4 5*
5×10^{-4}	− 77 6 ± 2 7***	+ 16 8 ± 1.2***	− 62 1 ± 13 5**	− 6 9 ± 1.2**	− 4 8 ± 2.0
10^{-3}				− 11 0 ± 1 7**	− 35 7 ± 9 1*

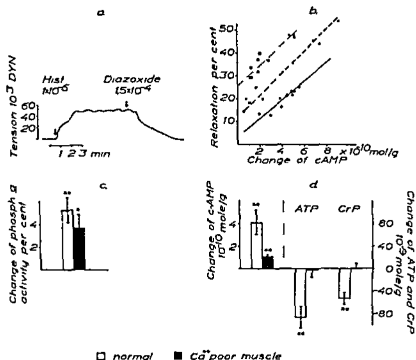


Fig 5a The effect of diazoxide (1 5 × 10 ⁴ g/ml) on histamine contracted bovine mesenteric artery

b Correlation between the degree of relaxation and the increase of cyclic AMP content after diazoxide ● --- ●, hydralazine ☐ --- ☐ and isoprenaline x — x in bovine mesenteric artery

Changes in (c) phosphorylase *a* activity, (d) the content of cyclic AMP, ATP and CrP of mesenteric artery 3 min after addition of diazoxide (1 5 × 10^{-4} g/ml) Mean ± S E M (n = 8) Statistical significance as in fig 1

effect being dependent on the presence of Ca^{++} (fig 5 D) The increasing effect on the cyclic AMP level was diminished (difference 3 0 ± 1 0 × 10^{10} mol/g, P < 0 05) in the Ca^{++}-poor preparations but still statistically significant (fig 5C)

Hydralazine (1 3 × 10^{-6} g/ml) produced a slow and moderate relaxation of the mesenteric artery It increased the cyclic AMP content (fig 6) The relaxing effect was correlated with the increase in cyclic AMP (r = 0 81, P < 0 01) (fig 5 B) In the Ca^{++}-poor vessel the cyclic AMP effect was increased The difference between the test with and without Ca^{++} was 1 4 ± 0 4 × 10^{10} mol/g (P < 0 01) Hydralazine increased the phospho rylase *a* activity and reduced the ATP content, the latter effect was Ca^{++}-dependent (fig. 6)

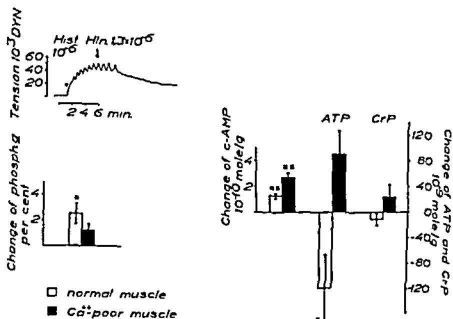


Fig 6 The upper part The effect of hydralazine (Hln 1 3 × 10^{-6} g/ml) on bovine mesenteric artery contracted by histamine (Hist 1 × 10^{-6} g/ml)
The lower part Changes in phosphorylase *a* activity and in the content of cyclic AMP, ATP and CrP of mesenteric artery 15 min after the addition of hydralazine (3 × 10^{-6} g/ml) Mean ± S E M (n = 6) Statistical significance as in fig 1

Neither diazoxide nor hydralazine had any significant effect on the PDE-activity of the muscle *in vitro* When added to the homogenate, however, the PDE-activity was moderately inhibited by diazoxide, the effect was dose-dependent (table 3) Hydralazine inhibited the PDE-activity in homogenate from the mesenteric artery only in high concentrations In lower concentrations an activation was observed (table 3)

Discussion

Papaverine, nitroglycerine, diazoxide and hydralazine are effective smooth muscle relaxing agents All the drugs increased the cyclic AMP content of smooth muscle The degree of relaxation and increase in the cyclic AMP level were correlated The drugs reduced the ATP-content, an effect not present in the Ca^{++}-poor preparation They stimulated though to a varying degree the phosphorylase *a* activity and the carbohydrate metabolism The mechanical and metabolic actions of these drugs showed a similarity with

regard to the action of adrenergic β receptor stimulation and the effect of exogenously added cyclic AMP (ANDERSSON 1972a & 1973a)

From the intestinal smooth muscle microsomal fractions were isolated that bound Ca^{++}, an effect that was increased by isoprenaline and cyclic AMP (ANDERSSON & NILSSON 1972) Papaverine and nitroglycerine produced a very marked inhibition of the PDE activity in these fractions (table 2 & 3) This initiated an investigation to determine whether the Ca^{++}-binding was also influenced Papaverine increased significantly the Ca^{++} binding of the fraction isolated at a sucrose gradient of 35–45 % (ANDERSSON & NILSSON 1972) Cyclic AMP and the drugs that elevate the cyclic AMP level may therefore produce a relaxing effect by stimulating a Ca^{++}-binding process in smooth muscle and thereby reduce free myo plasmic Ca^{++} The mechanism of this action of cyclic AMP is further discussed by ANDERSSON (1972b)

The possibility that the drugs produced relaxation of the smooth muscle by other mechanisms as well as by an increase in the cyclic AMP content must however be kept in mind Papaverine thus has a local anaesthetic action (GROLLMAN & GROLLMAN 1970) ABERG & ANDERSSON (1972) found that the relaxing effect of the local anaesthetic agent mepivacaine in rat portal vein and guinea pig taenia coli, was only partly attributable to its increasing action on the cyclic AMP content Mepivacaine inhibited the contracting effect of exogenously added Ca^{++} probably by reducing the permeability of the smooth muscle membrane to Ca^{++} The competitive inhibitory action of diazoxide on the contracting action of Ca^{++} (and Ba^{++} which can substitute for Ca^{++}) in vascular smooth muscle observed by WOHL *et al* (1968) indicates that a similar mechanism might contribute to the relaxing effect of this drug

Phosphodiesterase is a very complex enzyme or combination of enzymes The biochemistry of this complex is not yet clear From rabbit tissue MONN & CHRISTIANSEN (1971) isolated by the electrophoretic technique seven different forms of which up to four were simultaneously present in the same organ BLAVO *et al* (1970) and THOMPSON & APPLEMAN (1971) found PDE activities with different K_m and V_{max} when tested at different cyclic AMP concentrations AMER (1971) reported that phosphodiesterases with different K_m and V_{max} were interconvertible (PDE I and PDE II) and that the form with a high affinity (PDE II) was stimulated by some drugs such as adrenaline, an effect blocked by phentolamine

The action of papaverine and nitroglycerine on the PDE activity of the muscle *in vitro* and on the activity of different cell fractions was complex Papaverine and nitroglycerine stimulated cytoplasmic phosphodiesterase but inhibited the activity of the phosphodiesterase bound to the structural ele ments such as microsomes and mitochondria These tests may indicate the

existence of different PDE activities in smooth muscle It is of special interest that both papaverine and nitroglycerine had opposite effects on the cytoplasmic and structural bound PDE-activities Whether the drugs acted by changing one form of the enzyme into another or directly or in directly influenced the enzyme activity is not clear

The stimulating action of papaverine on the PDE activity of cytoplasm may explain why its increasing effect on phosphorylase *a* – a cytoplasmic enzyme – was weaker than that of isoprenaline despite the fact that the two drugs increased the total cyclic AMP content of rabbit colon to the same extent It may also explain why the increase in the phosphorylase *a* activity and relaxation were negatively correlated after papaverine (fig 3) These relations indicate a compartmentilization of cyclic AMP in smooth muscle a problem which will be further discussed in another paper (ANDERSSON 1972b)

The *manner in which diazoxide and hydralazine increase* the cyclic AMP level is not clear Diazoxide has been reported to inhibit the PDE activity in liver and skeletal muscle (SENFT *et al* 1968) WILKENFELD & LEVY (1969) suggested that the shift of the dose response curve of iso prenaline in rabbit ileum to the left by diazoxide was dependent on an inhibitory effect on the PDE activity The magnitude of the inhibitory effect of diazoxide in vascular smooth muscle found in this investigation is similar to that observed in heart muscle (MOORE 1968) The effect on the PDE activity is probably too weak however solely to explain the increase in the cyclic AMP content

Hydralazine inhibited the PDE activity of vascular smooth muscle only *in a very high concentration* (> 10^{-4} *g/ml) whereas it induced* relaxation of the isolated vascular smooth muscle in the concentration interval 10^{-7}–10^{-5} g/ml (ABLAD 1963) The mechanism of the increasing action of these drugs on the cyclic AMP level and its relation to Ca^{+} is the subject of further investigations

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REFERENCES

Amer S M Allosteric change in 3 5 cycl c adenos ne monophosphate phos phod esterase from rabb t t ssues In *Abstract I t Co f on Phys ol and Pl a r acol of Cycl c AMP* M lan 1971 p 8

Andersson, R Role of cyclic AMP and Ca^{++} in the metabolic and relaxing effects of catecholamines in intestinal smooth muscle *Acta physiol scand* 1972a, 85, 312–322.

Andersson, R Cyclic AMP and calcium ions in mechanical and metabolic responses of smooth muscles, influence of some hormones and drugs *Acta physiol scand* 1972 b, supplementum 382

Andersson, R Role of cyclic AMP and Ca^{++} in mechanical and metabolic events in isometrically contracting vascular smooth muscle *Acta physiol scand* 1973a, 87, 84–95

Andersson, R Relationship between cyclic AMP, phosphodiesterase activity, calcium and contraction in intestinal smooth muscle *Acta physiol scand* 1973b, in press

Andersson, R, Lundholm, L & E Mohme Lundholm Relationship between mechanical and metabolic events in vascular smooth muscle In *Proc Symp Physiol Pharmacol Vasc Neuroeffector Systems* S Karger, Basel 1971, 202–215

Andersson R & E Mohme Lundholm Metabolic actions in intestinal smooth muscle associated with relaxation mediated by adrenergic α and β receptors *Acta physiol scand* 1970, 79, 244–261

Andersson, R & K Nilsson Cyclic AMP and calcium in relaxation in intestinal smooth muscle *Nature* 1972, 238, 119–120

Beavo, J A, J G Hardman & E W Sutherland Hydrolysis of cyclic guanosine and adenosine 3 5 monophosphates by rat and bovine tissues *J biol Chem* 1970, 245, 5649–5655

Carsten M F Role of calcium binding by sarcoplasmic reticulum in the contraction and relaxation of uterine smooth muscle *J General Physiol* 1969, 53, 414–426

Ebashi, S & M Endo Calcium ion and muscle contraction *Progr Biophys Mol Biol* 1968, 18 123–183

Goldberg, N D, J Larner H Sasko and A G O Toole Enzymic analysis of cyclic 3, 5-AMP in mammalian tissues and urine *Anal Biochem* 1969, 28 523–544

Grollman, A & E F Grollman *Pharmacology and therapeutics* Lea & Febiger, Philadelphia, 1970

Kakiuchi, S & T W Rall The influence of chemical agents on the accumulation of adenosine 3 5 phosphate in slices of rabbit cerebellum *Mol Pharmacol* 1968, 4, 367–368

Kukovetz, W R & Poch, G Inhibition of cyclic 3, 5 nucleotidephosphodiesterase as a possible mode of action of papaverine and similarly acting drugs *Arch Pharmakol exp Path* 1970, 267, 189–194

Kukovetz W R G Poch & H Juan The role of phosphodiesterase inhibition and the mechanism of coronary dilation by drugs *Fourth Int Congr Pharm* 1969, Schwabe & Co Basel, pp 170

Levy, B & B E Wilkenfeld The potentiation of rat uterine inhibitory responses to noradrenaline by theophylline and nitroglycerine *Brit J Pharmacol* 1968, 34, 604–612

Monn, E & R O Christiansen Adenosine 3, 5 monophosphate phosphodiesterase, multiple molecular forms *Science* 1971, 173, 540–541

Moore, P F The effects of diazoxide and benzothiadiazine diuretics upon phosphodiesterase *Ann N Y Acad Sci* 1968, 150 256 260

Osnes, J B, T Christoffersen J Morland & I Øye Paper chromatography combined with phosphodiesterase treatment in the determination of radioactively labelled cyclic 3-5 adenosine monophosphate *J Chromatogr* 1972, 67, 139–147

Pöch, G Assay of phosphodiesterase with radioactively labeled cyclic 3, 5 AMP as substrate *Naunyn Schmiedebergs Arch Pharmak* 1971, 268 272–299
Rüegg J C. Smooth muscle tone *Physiol Rev* 1971, 51, 201–248
Senft, S, K Munske, G Schultz & M Hoffman Influence of hydrochlorothiazide and other sulfamoyl diuretics on the activity of 3', 5 AMP phosphodiesterase in rat kidney *Arch Pharmakol exp Path* 1968, 259 344–359
Simonis A M., E J Ariens & J van den Broeke Calcium as a factor in the action of spasmogens and nonspecific spasmolytics *Abstracts Fourth International Congress of Pharmacology* 1969, 417
Strauer, B E & H Avenhaus New results concerning the mechanism of the action of nitroglycerol In *Vascular smooth muscle* Ed E Betz. Springer, Berlin 1972 205–208
Triner, L., G G Nahas, Y Vulliemoz, N I A Overweg, M Verosky, D B Habif & S H Ngai Cyclic AMP and smooth muscle function *Ann N Y Acad Sci* 1971 185 458–476
Thompson, W J & M M Appleman Multiple cyclic nucleotide phosphodiesterase activities from rat brain *Biochemistry* 1971, 10 311–316
Weber, A, R Herz & J Reiss On the mechanism of the relaxing effect of fragmented sarcoplasmic reticulum *J Gen Physiol* 1963, 46, 679–702
Wilkenfeld, B E & B Levy The effects of theophylline, diazoxide and imidazole on isoproterenol induced inhibition of the rabbit ileum *Fed Proc* 1969, 28 741
Wohl, A J, L M Hausler & F E Roth Studies on the mechanism of antihypertensive action of diazoxide *In vitro* vascular pharmacodynamics. *J Pharmacol Exp Therap* 1967, 158 531–539
Wohl, A J, L M Hausler & F E Roth Mechanism of the antihypertensive effect of diazoxide *In vitro* vascular studies in the hypertensive rat *J Pharmacol Exp Therap* 1968 162, 109–114
Aberg G & R Andersson Studies on mechanical actions of mepivacaine (carbocaine®) and its optically active isomers on isolated smooth muscle Role of Ca^{++} and cyclic AMP *Acta pharmacol et toxicol* 1972 31, 321–336
Ablad B A study of the mechanism of the hemodynamic effects of hydralazine in man *Acta pharmacol et toxicol* 1963, 20 suppl 1 1–53

Acta pharmacol et toxicol 1973, 32, 337–344

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Trimethoprim in Human Saliva, Bronchial Secretion and Lung Tissue

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(Received April 18, 1972, Accepted May 25, 1972)

Abstract Following oral administration of trimethoprim (TMP) in doses varying from 4 0 to 11 2 mg TMP per kg body weight per 24 hours over a period of 1–5 days the concentration was determined in lung tissue (obtained by open biopsy), bronchial secretion or saliva, in a total of 51 cases A microbiological method was used to determine the concentration using *B pumilus* as test organism The concentrations of TMP found in the lung tissue bronchial secretion and saliva (6–13 hours after the last administration of TMP) were at a therapeutically active level (≥ MIC×5) with regard to the majority of those bacteria usually occurring in respiratory tract infections In 14 out of 17 patients TMP was found in the lung tissue in concentrations which were 1½ to 3½ times higher than the corresponding serum concentration In the other 3 patients the concentrations found were 4½ times 7 times and 14 times higher in the lung tissue than in the serum In the case of bronchial secretion 6 patients showed a TMP concentration which was ½ to 4 times the corresponding serum concentration while in 14 patients an examination of the saliva showed a TMP concentration which was 1–8½ times that of the corresponding serum concentration

Key words Trimethoprim tissue concentrations

Trimethoprim (2 4 diamino-5 (3,4,5-trimethoxylbenzyl) pyrimidine) in combination with sulphamethoxazole, has now been available for clinical use for several years The field of clinical application has been mainly in acute and chronic infections of the respiratory and urinary tract The clinical effect of trimethoprim (TMP) in combination with sulphamethoxazole (bactrim®, septrim®, eusaprim®) in infections of the respiratory tract has been the subject of several investigations (Drew *et al* 1967a & b, General Practitioner Research Group 1969, Hughes 1969a & b, Hughes *et al* 1969, Corbeel & Stenier 1970, Fierlafyn 1970, Marcic & Ulmer 1970, Pines 1970, Van Durme 1970) Only a few investigations have been made on the concentration of TMP in human tissue with the clinical dosage used In experiments on mice, TMP concentrations have been demonstrated in lung

tissue which are 10–17 times those of the corresponding serum concentrations (BUSHBY & HITCHINGS 1968), however, no studies have been published on the concentrations of TMP which can be obtained in human lung tissue and in bronchial secretion The aim of the present study was to examine the concentrations of TMP (F Hoffmann - La Roche & Co , Ltd , Basle, Switzerland) in lung tissue, bronchial secretion and saliva after oral administration of TMP in doses employed clinically

Material and Methods

Patients The material consisted of 51 cases with normal renal function evaluated by two determinations of the serum-creatinine These patients were divided into three groups as follows

1) 19 cases in whom the TMP concentration was determined in the saliva,

2) 15 cases in whom the TMP concentration was determined in the bronchial secretion 3) 17 cases in whom the TMP concentration was determined in the muscle and lung tissue In two cases the determination of the TMP concentration in the saliva and bronchial secretion was made several times in each patient Between each treatment series a period of 4 to 7 days elapsed without the administrat on of TMP

TMP dose TMP was given orally as tablets the dose varying from 4 0 to 11.2 mg TMP per kg body weight divided into 3 doses per 24 hours The duration of treatment was from 1 to 5 days before the TMP concentration was determined

Saliva The sample was obtained in the morning from the fasting patient 6–12 hours after the last administration of TMP After having emptied the oral cavity of saliva, the saliva production was collected during the next 2 minutes the patient making chewing movements

Bronchial secretion The secretion was aspirated through a bronchoscope during diagnostic or therapeutic bronchoscopy with the patient under general anaesthesia Only the secretion from the bronchi of the lower lobe was used In 2 tracheostomized patients suction was carried out through the tracheostomy cannula the intention was to collect secretion from the peripheral parts of the bronchial tree

Muscle and lung biopsy The tissue biopsies were obtained from patients who underwent thoracotomy The muscle biopsy was taken from the musculus latissimus dors the lung biopsy from lung tissue which had a macroscopically normal appearance and always from a lobe of the lung which was unaffected by cancer Both the muscle and lung tissue were dipped in physiological saline immediately after the biopsy was performed in order to reduce contamination of the surface of the tissue with blood

Control material In 3 cases the antibacterial effect of saliva bronchial secretion muscular tissue and lung tissue respectively were examined with regard to the test bacterium used, without any preced ng administration of TMP The t ssues and secretions examined were not found to have any antibacterial effect with regard to the test bacterium used

Bacteriological method All the samples were examined by the "disc plate diffus on method with *Bacillus pumilus* (laboratory strain) as test organism and peptone-free

beef extract agar containing 5 % horse blood as medium The plates were seeded with broth culture in dilution 1 10 after incubation for 24 hours at 37° The principles followed for the determination of concentrations were those previously described (THAMDRUP ROSDAHL *et al* 1969, LYKKEGAARD NIELSEN & HANSEN 1972) The serum concentration of TMP was determined at intervals of ½ to 1½ hours twice before and twice after the biopsy sample was obtained or the secretion was collected The serum samples were examined undiluted The saliva was examined after the addition of pooled human serum in a dilution of 1 3 Bronchial secretion and biopsy specimens from the lung and muscle tissue were homogenized in a modified Potter Elvehjem tissue homogenizer after addition of pooled human serum in dilutions of 1 2 or 1 3 (in 2 cases a higher dilution) depending on the volume of the biopsy specimen

In 3 cases, 2 parallel determinations were made, the material being homogenized by using both the Potter Elvehjem technique and the microblender (OMNI mixer) A good agreement was found as the two methods of homogenization showed difference in the concentrations of at most 15 %

Results

Table 1 shows the concentration of TMP in the muscle and lung tissue, together with the corresponding serum concentration, in relation to the TMP administered In 14 of the 17 cases, the TMP concentration in the lung tissue was found to be 1½–3½ times greater than the serum concentration measured before and after the biopsy specimen was obtained Of these 14 patients, 11 were treated with TMP for only 24 hours, while the other 3 patients were treated for 2–4 days In 3 of the 17 cases, the TMP concentration in the lung tissue was found to be 4½, 7 and 14 times greater than the corresponding serum concentration after TMP treatment for 1, 3 and 2 days, respectively In all 17 patients, the TMP concentration in the muscle tissue was found to be at about the same level as the corresponding serum concentration

Table 2, which lists the TMP concentrations in the bronchial secretion, shows that 2 patients underwent 7 and 4 investigations, respectively, after varying periods of treatment In the 2 patients with tracheostomy and bronchorrhoea, the TMP concentrations found in the bronchial secretion were rather consistently 1–2 times that of the corresponding serum concentration In the other 4 patients, the TMP concentrations in the bronchial secretion were found to be ½–4 times that of the corresponding serum concentration

Table 3 shows the concentrations of TMP in saliva In 1 patient, who underwent 4 investigations after varying periods of treatment, the TMP concentrations in the saliva were found to be at the same level as the corresponding serum concentration In the other 16 investigations, the TMP concentrations in the saliva were 1–8½ times higher than the corresponding serum concentration

Concentrations of TMP in serum, lung tissue and muscular tissue 9–13 hours after last intake of TMP perorally Serum concentrations were determined at intervals of ½–1½ hours before and after biopsy

Case No	Age	Sex	Body weight	Diagnosis	Intake of TMP in mg/kg body weight per 24 hrs	Days of intake	Weight of lung biopsy (g)	Hours between TMP intake and biopsy of lung	TMP-concentration in serum before and after time of biopsy of lung µg TMP/ml serum	Muscle biopsy µg TMP/g	Lung biopsy µg TMP/g	Lung/Serum Factor µg TMP/g / µg TMP/ml	Muscle/Serum Factor µg TMP/g / µg TMP/ml
1	66	M	121	C pulm	4.0	1	0.2	13	1.9 (-) (×) 1.4 (-)		2.9	2	
2	63	M	91	–	5.3	2	0.4	12½	1.9–1.4 (×) 1.5–1.3	1.5	2.9	2	1
3	66	F	78	–	6.1	4	0.3	10	4.2–3.2 (×) 3.2–3.2	1.9	10.7	3½	½
4	52	M	78	–	6.2	1	0.5	9½	1.8–1.5 (×) 1.5–1.3	0.8	3.2	2	½
5	53	F	77	–	6.3	1	0.5	10½	(-) 1.7 (×) 1.7–1.6	1.7	2.9	1½	1
6	45	M	75	–	6.4	1	0.6	10½	(-) 2.0 (×) 1.9–1.6	2.0	4.9	2½	1
7	32	M	72	Pneumo thorax	6.7	1	0.5	10½	2.5–2.0 (×) 1.9–2.1	1.6	3.6	2	1
8	72	M	66	C pulm	7.3	1	0.4	9¼	3.5–2.9 (×) 2.7 (-)	2.0	3.8	1½	½
9	74	F	64	Hernia diaphragm	7.5	1	0.4	12	3.1–2.8 (×(2.7–2.7	2.9	5.7	2	½
10	21	M	63	Tumor mediastini	7.6	1	0.2	9½	1.8–1.5 (×) 1.5–1.4	1.4	3.6	2½	1
11	59	F	62	Hypernephr metast ad pulm	7.7	1	0.3	9½	6.9–6.5 (×) 6.5–6.3	7.5	23	3½	1
12	60	M	61	C pulm	7.9	1	0.5	10	2.2–1.6 (×) 1.5–(-)	1.5	3.6	2½	1
13	44	F	59	–	8.1	1	0.4	9½	1.9–1.8 (×) 2.0–1.7	1.6	7.2	3½	1
14	75	M	76	–	9.5	3	1.1	13	5.2 (-) (×) 3.6–3.4	3.5	8.4	2½	1
15	43	M	76	Hernia diaphragm	9.5	3	0.4	9½	6.8–6.1 (×) 5.9–5.7	6.9	43.5	7	1
16	25	F	51	Cystis pulm	9.6	2	0.3	10½	2.9–2.3 (×) (-)–(-)	2.1	32	14	1
17	38	F	43	C pulm	11.2	1	0.4	11	(-) 2.6 (×) 2.3–2.1	1.7	10.8	4½	½

(×) time of biopsy taking of lung tissue

(-) No determination made

Table 2

Concentrations of TMP in serum and bronchial secretion (br secr) 9 13 hours after last intake of TMP perorally Serum concentrations were determined at intervals of ½–1½ hours before and after collection

Case No	Age	Sex	Body weight	Diagnosis	Infection of the br secr	Intake of TMP in mg/kg body weight per 24 hrs	Days of TMP intake	Weight of the br secr specimen (g)	Hours between TMP intake and collection of br secr	TMP conc in serum before and after time of collection of br secr µg TMP/ml serum	br secr µg TMP/g	br secr / serum Factor µg TMP/g / µg TMP/ml	Remarks	
18	43	M	93	Frac costar	+	5 2	1	0 5	9	2 5–2.3 (×) 2.3	3 4	1½	5 consecutive days	Samples from the same patient
19	43	M	93	–	+	5 2	2	0 9	7½	2 8–2 8 (×) 2.7	1 8	½		
20	43	M	93	–	+	5.2	3	1 1	9	3 1–3 1 (×) 3 0	4 4	1½		
21	43	M	93	–	+	5 2	5	1 0	8	1 5–1 3 (×) 1 4	3 0	2		
22	73	M	78	–	–	6 1	1	0 4	8½	3 1–3 0 (×) 2 9	2.5	1	5 consecutive days	Samples from the same patient
23	73	M	78	–	–	6 1	2	1 3	7½	3 6–3 4 (×) 3 4	3 6	1		
24	73	M	78	–	–	6 1	3	0 8	8½	3 3–3 3 (×) 3 2	2 8	1		
25	73	M	78	–	–	6 1	5	1 1	8	3 5–3 7 (×) 3 9	3 8	1		
26	73	M	78	–	+	6 1	2	0 3	12	2 0–1 9 (×) 1 8	2 5	1½		
27	73	M	78	–	–	6 1	3	0 2	10½	2 0–1 9 (×) 1 9	1 3	½		
28	73	M	78	–	–	6 1	3	0 2	9	3 7–3 6 (×) 3 6	2 4	½		
29	38	M	64	Bronchitis chr	+	7 5	1	1 4	13	2.0–1 9 (×) 1 7	3 0	1½		
30	74	F	64	Hernia diaphragm		7 5	1	0 9	12½	(–)–2 6 (×) 2.4	8 0	3		
31	64	F	57	C pulm Astma	–	8 4	2	0 3	12	(–)–3 3 (×) 2.9	1 8	½		
32	20	F	55	Bronchitis chr		8 8	1	1 1	11½	3 2–3 3 (×) 3 1	12.3	4		

(×) time of collection of bronchial secret on (–) No determination made

Table 3.

Concentrations of trimethoprim (TMP) in saliva 6–12 hours *after last intake* of TMP perorally Serum concentrations were determined at *intervals of ½–1½* hours before and after collection

Case No	Age	Sex	Body weight	Intake of TMP in mg/kg body weight/24 hrs	Days of TMP intake	Weight of the saliva specimen (g)	Hours between TMP intake and collection of saliva	TMP concentration in serum before and after *time of* collection of saliva µg TMP/ml serum	Saliva µg TMP/g	Saliva/ Serum Factor µg TMP/g / µg TMP/ml	Remarks	
33	57	M	105	4 6	1	1 3	8	(-) 2.3 (X) (-) 1 5	3 9	2		
34	60	M	99	4 9	1	0 3	9	(-) 2 1 (X) (-) 1 7	1 9	1		
35	63	M	95	5 0	1	0 8	9	(-) 2.5 (X) (-) 2.2	2.9	1		
36	43	M	93	5.2	1	1 0	9	2 5–2 3 (X) 2 3 (-)	5 4	2½		
37	44	M	85	5 6	1	0 5	9	(-) 2 4 (X) (-) 2.1	4 3	2		
38	73	M	78	6 1	1	0 8	8½	3 1–3 0 (X) (-) 2.9	1 5	½	3 con	Samples
39	73	M	78	6 1	2	0 8	12	2 0–1 9 (X) (-) 1 8	2.3	1	secutive	from the
40	73	M	78	6 1	3	0 6	10	2 0–1 9 (X) 1.9–1 8	2.2	1	days	same
41	73	M	78	6 1	3	0 6	9	3 7–3 6 (X) 3 6–3 5	3 0	1		patient
42	24	M	72	6 6	1	1 6	11½	2.4–2 2 (X) 2 1–2.0	3 0	1½		
43	72	M	66	7 3	1	0 6	8	(-)–3 5 (X) 2 9–2.7	14 0	4½		
44	74	F	64	7 5	1	0 6	9	3 9–3 8 (X) 3 1–2.8	13 2	4		Samples
45	74	F	64	7 5	1	0 6	10	(-)–3 3 (X) (-) 2 6	27	8½		from the
46	21	M	63	7 6	1	0 5	8	(-)–1 8 (X) 1 5–1 5	4 2	3		same
47	59	F	62	7 7	1	0 3	6	(-)–(-) (X) 6 9–6 5	39	5½		patient
48	60	M	61	7 9	1	0 9	8½	(-)–3 5 (X) 2 9–2 4	16 5	4½		Samples
49	60	M	61	7 9	1	1 0	8	(-)–2.8 (X) 2.2–1 6	20 0	8		from the
50	79	F	58	8 3	1	0 5	9	(-)–4 7 (X) (-) 4 3	12 0	2½		same
51	25	F	51	9 6	2	0 8	8	( )–3 5 (X) 2 9–2 3	7 2	2		patient

(X) time of collection of saliva (-) No determination made

Discussion

In evaluating the results, allowance must be made for conditions such as varying activity for the secretion and varying protein content in the samples examined as well as the possibility of infection of the bronchial secretion and of the lung tissue In the present study, no determination was made of the protein content of the samples examined On the basis of the present material it was impossible to determine whether infection of the respiratory tract plays a role in the excretion of TMP in the bronchial secretion

The technique of homogenization used is regarded as being effective in view of the good agreement between the results obtained by using two different homogenization procedures

The tables show that there is a great variation in the concentrations of TMP found in lung tissue, while the concentrations in muscle tissue are very constant in relation to the corresponding serum concentration It might be mentioned that no histological examination was made of the lung biopsies obtained but all the biopsies were taken from lung tissue which had a macroscopically normal appearance A possible reason for the varying TMP content in the lung biopsies might be diseases in the lung tissue, which cannot be recognized macroscopically, including for example varying degrees of pulmonary fibrosis, emphysema and infection On the basis of the present investigation, it is impossible to decide whether TMP accumulates in human lung tissue

According to studies of the minimum inhibitory concentrations (MIC) of TMP on the bacterial pathogens of the respiratory tract (DARRELL *et al* 1968, HUGHES *et al* 1969 VAN DURME 1970), the TMP concentration in the present investigation was at a therapeutically active level ($\geq$ MIC $\times$ 5, with an MIC for the bacteria of $\leq$ 0 5) and would be expected to exert effective bacteriostasis with regard to the bacteria usually occurring in respiratory tract infection and pneumonia Regarding certain *Proteus* species, however, it is doubtful whether the concentrations found are at a therapeutic level In this connection however it should be mentioned that the concentrations found represent minimum concentrations since the investigations were made 8–12 hours after the last administration of TMP It should also be mentioned that *Pseudomoans aeruginosa* is resistant to TMP

REFERENCES

Bushby S R M & Hitchings G H Trimethoprim a sulphonamide potentiator *Brit J Pharm Chemother* 1968 33 72–90

Corbeel L & P Stenier Etude clinique en double aveugle de l association sulfamethoxazole/trimethoprime dans les infections pulmonaires chroniques 5ème Congres International des Maladies Infectieuse

Darrell, J H, L P Garrod & M P Waterworth Trimethoprim laboratory and clinical studies *J clin Path* 1968, 21, 202–209

Drew, C D M, D T D Hughes & G C Jenkins Long term treatment of chest infections with a combination of trimethoprim and sulphonamide the clinical, bacteriological & haematological effects 5th Internat Congress on Chemotherapy Vienna 1967a

Drew, C D M, D T D Hughes, A S E Fowle & M A Cassell Effective treatment of chronic bronchitis with short term trimethoprim and sulphamethoxazole 5th Internat. Congress on Chemotherapy, Vienna, 1967b

Fierlafyn, E Etude clinique a double insu d un chimio-therapique bactericide en medicine generale 5ème Congres International des Maladies Infectieuses, Vienne 1970

Report from the General Practitioner Research Group on the Bactrim/Ampicillin Trial *The Practitioner* 1969, 203, 817–819

Hughes, D T D, C D M Drew, T B W Johnson & J D Jarvis Trimethoprim and sulphamethoxazole in the treatment of chronic chest infections *Chemotherapy* 1969, 14 151–157

Hughes, D T D The successful treatment of pneumonia with a combination of trimethoprim and sulphamethoxazole *6th International Congress on* Chemotherapy Tokyo, 1969a

Hughes, D T D Single blind comparative trial of trimethoprim sulphamethoxazole and ampicillin in the treatment of exacerbations of chronic bronchitis *Brit med J* 1969b, 4, 470–473

Lykkegaard Nielsen, M & I Hansen Trimethoprim in human prostatic tissue and prostatic fluid *Scand J Of Urol Nephrol* 1972, in press

Marcic, I & W T Ulmer Die Langzeitbehandlung der chronischen Bronchitis mit dem Breitband Chemotherapeuticum "Bactrim" *Pneumonologie* 1970, 142 59–66

Pines A Trimethoprim and sulphamethoxazole in the prevention of purulent exacerbations of chronic bronchitis 5th International Congress on Infectious Diseases, Vienna, 1970

Thamdrup Rosdahl V, V Vejlsgaard N Rosdahl & R Vejlsgaard A micromethod for determination of antibiotics in serum *Dan med Bull* 1969 16 133–139

Van Durme P Experimental research concerning the value of a new antibacterial combination in the therapy of acute infections of the lungs and respiratory tract. 5ème Congres International des Maladies Infectieuses Vienne, 1970

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Effect of Acetylcholine on Isolated Cat and Rabbit Heart Ventricle Preparation

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Abstract The atria and the upper part of the intra ventricular septum were removed from rabbit and cat heart, and the ventricular preparations were perfused *in vitro* with Krebs s solution Preparations from twenty cats and 15 rabbits were studied for their sensitivity to acetylcholine In the isolated rabbit ventricular preparations, acetylcholine in doses of 0 01–0 1 µg caused a very weak negative inotropic and a chronotropic effect Higher doses caused a dose dependent standstill The cat ventricle preparations were rather insen sitive to acetylcholine When studied against noradrenaline induced increases in heart rate and force of contraction acetylcholine in these preparations, also, caused a negative inotropic and chronotropic effect In both the cat and rabbit preparations, the effect of acetylcholine was blocked by atropine 0 1 µg/ml in the perfusing solution After pretreatment with atropine, acetylcholine (50–100 µg) caused a positive inotropic and chronotropic effect that was blocked with propranolol 0 1–0 2 µg/ml This effect of acetylcholine was not obtained in preparations from reserpine treated animals

It was concluded that the cat and rabbit ventricle myocardium contains receptors to acetylcholine and that stimulation of these cause a negative inotropic and chronotropic effect Moreover, acetylcholine may release nor adrenaline from the adrenergic nerve fibres The difference in sensitivity to acetylcholine between the two species may indicate a difference in the signifi cance of parasympathetic nerve stimulation to the ventricular myocardium in the two species

Key words Ventricular myocardium – acetylcholine

The mammalian heart is controlled by inhibitory cholinergic fibres and excitatory adrenergic fibres The cholinergic fibres innervate the SA node and the atrium, but are not believed to innervate the ventricles (Burnstock 1969) Adrenergic fibres, however, are found in all parts of the heart

Napolitano *et al* (1965) studied the innervation of canine hearts that had been excised and reimplanted This technique guarantees complete

extrinsic denervation of the heart Electron microscopy of these hearts revealed neural elements in the ventricles as well as in the auricles It was not known whether these fibres were cholinergic or adrenergic in origin As the fibres were unaffected by heart excision and reimplantation they must have their cell bodies within the heart itself

EHINGER *et al* (1968 & 1970) found adrenergic as well as acetylcholine esterase containing fibres in the hearts of mice, rats, guinea pigs, rabbits, and cats The adrenergic nerve fibres were demonstrated by the fluorescence method of FALCK *et al* (1962) and the cholinesterase containing fibres according to a modified Koelle technique Both kinds of fibres were found in all parts of the heart from these species However, the fibres were more abundant in the atria than in the ventricles In the valves, it could be shown that adrenergic and cholinesterase containing fibres run closely parallel to each other

Recent studies by EKSTROM (1970) have also shown that large amount of cholineacetylase can be isolated from the cat and rabbit heart ventricles An acetylcholine like substance has also been isolated from the atria and ventricles of the rabbit (DAY 1956), rat (ROTHSCHUH 1954) and dog (WASILEWSKA-OZIUBINSKA 1970) Thus there are good reasons for believing that the mammalian heart ventricles are innervated by cholinergic fibres

The results of physiological studies are contradictory BOLTON (1966) and SARNOFF (1960) failed to show any function of the cholinergic nerve fibres in the ventricular myocardium of guinea-pigs and dogs Other investigators report a cardiostimulatory as well as a cardioinhibitory effect when the vagus was stimulated (for references, see LEVY 1971) and LEVY & ZIESKE (1969) The purpose of this study was to re investigate the effect of acetyl choline on the mammalian ventricular myocardium Spontaneously beating ventricular preparations from cats and rabbits were used

Methods

20 cats and 15 rabbits were used The rabbits were stunned by a blow on the head and bled to death The cats were anaesthetized with pentobarbital (mebumalum NFN) (40 mg/kg intraperitoneally) and bled to death by cutting the carotid arteries The hearts were excised and quickly connected to a perfusion apparatus of the kind described by ANDEN *et al* (1964) and perfused according to Langendorff with Krebs's solution warmed to 37° The solution was gassed with a mixture of oxygen (95 %) and carbon dioxide (5 %) The flow was kept constant (about 20 ml/min) by means of a Sigma motor pump (model T8) Coated steel wires (0 1 mm) were hooked into the heart at the base of the ventricles and one in the apex for ECG recording

The atria and the upper part of the septum were removed to get a preparation with an idio-ventricular pacemaker This preparation was previously studied by BENFORADO (1958) After the experiments different parts of the hearts were pricked with a blunt needle to localize roughly the ectopic focus

Solutions of agonists were injected into the perfusion solution close to the heart Antagonists were either injected into the perfusion solution two minutes before the injection of the agonists or perfused into the perfusion solution by means of a Braun infusion pump The effects of acetylcholine and noradrenaline were studied in the following doses injected in 0 1 ml of solution 0 001, 0 01 0 10, 1 00 and 10 0 µg In 5 cat and 3 rabbit preparations 50 and 100 µg were also injected Reserpine (3 mg/kg) was given intraperitoneally to three cats 15–20 hours before the experiment

Compounds used acetylcholine iodide (Calbiochem) l noradrenaline bitartrate (Sigma) propranolol HCl (ICI, Macclesfield, Cheshire) physostigmine salicylate (E & H Smith Ltd Edinburgh) atropine sulphate reserpine (serpasil®, Ciba)

Results

Removal of the atria caused a decrease in heart rate in the isolated rabbit and cat hearts A further reduction in the heart rate was obtained after the elimination of the upper part of the intraventricular septum Rabbit hearts beat at the rate of 144 ± 7 beats/min After removal of the SA node, the heart rate decreased to 76 ± 6 beats/min The ventricular preparation beat at a rate of 33 ± 3 beats/min Corresponding figures for the cat hearts were 156 ± 8, 108 ± 8, and 55 ± 8 beats/min

Removal of the atria led to the disappearance of the P-wave in the ECG After removal of the upper part of the septum, the ECG changed dramatically After cutting the upper part of the intraventricular septum, a period with escaped ventricular beats was often observed The isolated ventricle preparation was allowed to stabilize for 10–15 minutes, during which time the ventricular rhythm stabilized A rough localization of the focus was done by pricking the heart with a blunt needle In the rabbit heart preparations, the ectopic focus could be localized in this way to the lower part of the right ventricle In the cat heart preparations the focus appeared in the lower part of one or other ventricle In the isolated rabbit as well as in the cat hearts, acetylcholine in doses of 0 1–1 0 µg caused a negative chronotropic and negative inotropic effect In the isolated rabbit ventricle preparations, acetylcholine in doses of 0 01–0 1 µg caused a very weak negative inotropic and chronotropic effect Higher doses caused a dose-dependent ventricular standstill (fig. 1) The ventricle preparations were more sensitive to acetylcholine than was the whole heart preparation In 13 of 15 ventricle preparations, an effect was obtained with 0 01 µg acetylcholine This dose had no effect on the whole heart

Most cat ventricle preparations were rather insensitive to acetylcholine In 3 out of 20 preparations acetylcholine, 0 1 µg, caused a weak negative inotropic and chronotropic effect No standstill of the ventricle, as observed for the rabbit preparations, was obtained In 6 of the insensitive cat ventricle preparations acetylcholine was tested against a noradrenaline induced increase in the force of contraction and heart rate In these preparations,

15 g A
10
5
0
Ach 0.01 µg
10 sek

15 g B
10
5
0
Ach 0,10 µg
10 sek

15 g C.
10
5
0
Ach 1,0 µg
10 sek

Fig 1. Isolated rabbit heart ventricle preparation. Effect of acetylcholine.
A. Acetylcholine 0.01 µg
B. Acetylcholine 0.1 µg
C. Acetylcholine 1.0 µg

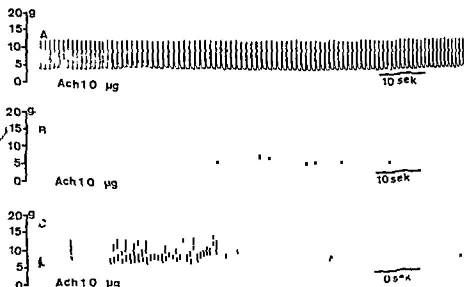


Fig 2. Isolated cat heart ventricle preparation. Effect of acetylcholine 1.0 µg/ml.
A. Without noradrenaline infusion
B. During infusion of noradrenaline 0.01 µg/ml
C. During infusion of noradrenaline 0.1 µg/ml

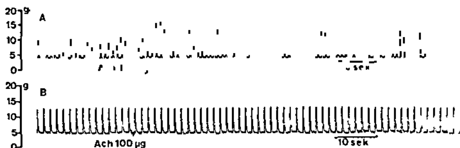


Fig 3 Isolated cat heart ventricle preparation
A Effect of acetylcholine (100 μg) after pretreatment with atropine (10 μg) injected into the perfusing solution 2 minutes before the injection of acetylcholine
B Effect of acetylcholine (100 μg) after pretreatment with atropine as described in A The animal pretreated with reserpine 3 mg/kg 15 hours before the test

acetylcholine caused a negative inotropic and chronotropic effect (fig 2) The sensitivity to noradrenaline was the same for the ventricle preparations as for the whole heart

In the cat as well as in the rabbit heart preparations, the negative inotropic and chronotropic effect of acetylcholine was readily blocked with atropine infused into the perfusion solution to a final concentration of 0 1 μg/ml In the rabbit ventricle preparations, physostigmine (10 μg/ml) added to the perfusion solution potentiated the effect of acetylcholine In the cat ventricle preparations insensitive to acetylcholine, no potentiation was obtained after the addition of physostigmine but when acetylcholine was studied against noradrenaline induced increase in ventricular force and rate, physostigmine increased the effect of acetylcholine

The effect of noradrenaline was reduced or completely blocked with propranolol infused into the perfusing solution in a final concentration of 0 1–0 2 μg/ml In 5 cat and 3 rabbit ventricle preparations pretreated with atropine (10 μg injected into the perfusing solution) acetylcholine in high doses (50 and 100 μg) caused a positive inotropic and chronotropic effect (fig 3) that was blocked with propranolol infused into the solution in a final concentration of 0 1–0 2 μg/ml This positive inotropic and chronotropic effect was not obtained in 3 hearts from reserpine treated cats (fig 3)

Discussion

The evidence for the elimination of the SA and AV-node is anatomical since the parts of the heart containing these structures were removed The change in the heart rate after the excision of the atria and the upper part of the septum also indicates that the SA- and AV nodes were absent

Moreover, by simultaneous recording, of epicardial ECG, the idoventricular focus was roughly localized By pricking different part of the ventricle, an ECG was obtained that was similar in shape to that obtained from the idioventricular focus The shape of the ECG also indicated that it was derived from a part of the ventricle other than the AV-node

In the present study, a clear difference between the cat and rabbit heart preparations was observed No effect of acetylcholine could be obtained in most cat preparations until the tone was raised by the infusion of noradrenaline This agrees well with the results obtained by KISSLING *et al* (1972) in the isolated cat papillary muscle preparation In preparations from cats pretreated with reserpine, these authors obtained a negative inotropic effect of endogenous acetylcholine released by high voltage stimuli only when contractility was increased by isoprenaline or noradrenaline Similar observations has been made for the dog heart ventricle *in situ* (for references, see LEVY 1971)

The difference in sensitivity to acetylcholine between the rabbit and cat heart ventricle is not easily understood The heart ventricle preparations from rabbits anaesthetized with pentobarbital before bleeding to death did not behave differently from those obtained in the manner described in the methods

The negative inotropic effect of acetylcholine obtained in noradrenaline induced increase in heart rate and force of contraction may be an effect of acetylcholine on the cAMP level In myocardial pieces from dog hearts MURAD *et al* (1962) obtained a 30 % decrease in the cAMP level after treatment with acetylcholine and carbachol A decrease in the cAMP level was also reported by LEE *et al* (1971) in rat and calf slices after treatment with acetylcholine Acetylcholine lowered the basic level of cAMP as well as the increase in cAMP obtained by isoprenaline and glucagon Whether the decrease in cAMP is due to a decrease in the adenylcyclase activity or to an increase in the phosphodiesterase activity is not clear

The negative inotropic and chronotropic effect of acetylcholine was blocked with atropine In both the isolated rabbit and cat ventricle preparation, high doses of acetylcholine in the presence of atropine produced a positive inotropic and chronotropic effect As this was blocked by a β receptor blocking agent and was absent in hearts depleted of catecholamines, this effect may be caused by noradrenaline The dose of reserpine used to cause the depletion is known to rule out the histological appearance of adrenergic structures (CARLSSON 1966) It is well known that acetylcholine releases noradrenaline from peripheral stores (RICHARDSON & WOOD 1959, ANGELAKOS & BLOOMQUIST 1965, LOFFELHOLTZ 1967)

The results obtained in this study show that acetylcholine causes a negative inotropic and chronotropic effect in the rabbit and cat heart

ventricle preparation They also indicate that the sympathetic and parasympathetic nervous system may interfere both at the cellular and at the neuronal level in the heart ventricle When the vagus is stimulated, released transmitter may act on both the muscle cells and on the adrenergic nerve fibres The rabbit preparations was far more sensitive to acetylcholine than the cat preparation, this may indicate differences in importance of parasympathetic nerve stimulation of ventricular myocardium in the two species

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REFERENCES

Angelakos, T & E Bloomquist Release of norepinephrine from isolated hearts by acetylcholine *Arch int Physiol* 1965 **73** 397–402

Andén N E H Corrodi M Ettles E Gustafsson & H Persson Selective uptake of some catecholamines by the isolated heart and its inhibition by cocaine and phenoxybenzamine *Acta pharmacol et toxicol* 1964 **21** 247–259

Benforado J M A depressant effect of acetylcholine on the idioventricular pace maker of the isolated perfused rabbit heart *Brit J Pharmacol* 1958 **13** 415–418

Bolton T B & C Raper Innervation of domestic fowl and guinea pig ventricles *J Pharm Pharmacol* 1966 **18** 192 193

Burnstock G Evolution of the autonomic innervation of visceral and cardiovascular systems in vertebrates *Pharmacol Rev* 1969 **21** 247–324

Carlsson A Drugs which block the storage of 5 hydroxytryptamine and related amines In *Handbuch der experimentellen Pharmakologie* Ed W Esparmer 1965 **19** 529 592.

Day M The release of substances like acetylcholine and adrenaline by the isolated rabbit heart *J Physiol* 1956 **134** 558 568

Ehinger B B Falck, H Persson & B Sporrong Adrenergic and cholinesterase containing neurons of the heart *Histochemie* 1968 **16** 197 205

Ehinger B B Falck & B Sporrong Possible axo axonal synapses between peripheral adrenergic and cholinergic nerve terminals *Z Zellforsch* 1970 508–521

Ekstrom J Distribution of choline acetyltransferase in the hearts of mammals *Acta physiol scand* 1970 **80** 73–78

Falck B N A Hillarp G Thieme & A Torp Fluorescence of catecholamines and related compounds condensed with formaldehyde *J Histochem Cytochem* 1962 **10** 348–354

Kissling G K Reutter G Sieberg H Ngugen Duong & R Jacob Negative inotrope von endogenem Acetylcholin beim Katzen und Hunerventrikelmyokard *Pflugers Arch* 1972 **333** 35–50

Lee T P J F Kuo & P Greengard Regulation of myocardial cyclic AMP by isoproterenol glucagon and acetylcholine *Biochem biophys Res Commun* 1971 **45** 991 997

Levy M N & H Zieske Comparison of the cardiac effects of vagus nerve stimulation and of acetylcholine infusion *Amer J Ph*

Levy, M N Sympathetic parasympathetic interactions in the heart *Circulat Res* 1971, 29 437–445
Loffelholtz, K Untersuchungen über die Noradrenalin Freisetzung durch Acetylcholin am perfundierten Kaninherzen *Naunyn Schmiedebergs Arch exp Path Pharmak* 1967, 258, 108–122.
Murad F, Y M Chi, T W Rall & E W Sutherland Adenyl cyclase III The effect of catecholamines and choline esters on the formation of adenosine 3, 5 phosphate by preparations from cardiac muscle and liver *J biol Chem* 1962 237, 1233–1238
Napolitano, L M V L William C R Hanlon & T Cooper Intrinsic innervation of the heart *Amer J Physiol* 1965, 208, 455–458
Richardsson, J A & E F Woods Release of norepinephrine from the isolated heart. *Proc Soc exp Biol (N Y)* 1959, 100 149–151
Rotschuh K E Herzmuskeleigene Acetylcholin II Mitteilung Der normale Acetylcholin Gehalt der Vorhofs und Kammermuskulatur beim Frosch und bei der Ratte *Pflugers Arch ges Physiol* 1954, 258, 481–488
Sarnoff, S J, S K Brockman J P Gilmore, R J Linden & J H Mitchell Regulation of ventricular contraction Influence of cardiac sympathetic and vagal nerve stimulation on atrial and ventricular dynamics *Circulat Res* 1960, 8 1108 1122
Wasilewska Oziurbinska, E Distribution of acetylcholine in the structures of dog heart ventricles *Acta physiol pol* 1970, 21, 255–259

Acta pharmacol et toxicol 1973, 32, 353–357.

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Fatal Digitalis Poisoning

By

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Abstract Two cases of digitalis poisoning are described Case 1, a man, aged 40 years, swallowed for suicidal purposes about 50 digitalis tablets, each containing 50 mg standardized digitalis leaf, digitalis purpurea (0 5 i u.) Death occured about 8 hours after the intake A quantitative determination of digitalis glycoside in the gastric contents, and in the contents of the small intestine, blood and heart was performed with xanthydrol Case 2, a child, 11 days old because of a congenital heart disease was treated with digoxin ½–1 hour before death the child was given 0 7 mg digoxin intravenously A quantitative determination of digoxin was performed with xanthydrol in the liver, heart and lung and by the fluorometric method in the liver, heart kidney, urine and blood

Key words Digitalis – tissue concentrations – lethal poisoning

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Quantitative determination of digitalis glycosides in autopsy material

Because of the lack of suitable analytical methods only few cases have been reported, in which the concentration of digitalis glycosides have been determined in autopsy material after fatal poisoning. In the following, two cases of fatal digitalis poisoning analysed in our department are described

Methods and Results

The methods used in the present cases are based with only a few modifications on a colorimetric determination of digitoxin and digoxin with xanthydrol described by JELLIFE (1966) and on a fluorometric determination of digoxin described by JELLIFE & STEPHENSON (1969)

The colorimetric determination of digitalis glycosides with xanthydrol

Extraction One portion of organ or tissue, homogenized if required, is extracted three times with about twice its volume of chloroform at pH 4 The combined chloroform extracts are washed three times with 1 N NaOH and once with water (5 ml/100 ml

of chloroform extract) The washed chloroform extracts are dried with anhydrous NA_2SO_4 filtered and evaporated to dryness in a rotary evaporator at 50°

Thin layer chromatography Purification and identification of the isolated glycoside is attained by thin layer chromatography according to JELLIFE (1966) If the first elution from plate (see later) gives an impure eluate re-chromatography is performed using chloroform methanol (88 12) as solvent system (BIČAN FIŠTER & MERKAS 1969) Only the latter system gives a definite separation between digitoxin and gitoxin

Elution The zone containing the glycosides is scraped off and eluted with 3 × 3 ml of the chloroform methanol mixture (1 1) shaken thoroughly each time for about 15 min The supernatant is drawn off after centrifugation and the eluates are evaporated to dryness The residue is then dried at 105° for 10 minutes (BIČAN FIŠTER & MERKAS 1969) before the colorimetric determination is performed with xanthydrol which yields a red colour with 2 desoxy sugars

The colorimetric determination and the calculation are performed according to JELLIFE (1966)

The fluorometric determination of digoxin

Extraction 10 g of organ or tissue homogenized if required is extracted with 200 ml of methanol The supernatant is drawn off after centrifugation The extraction is repeated with 100 ml of methanol The combined methanol extracts are then evaporated to dryness in a rotary evaporator at 50° after adding 150 ml of chloroform

The determination is continued as described by JELLIFE & STEPHENSON (1969) but using an emission wavelength of 494 mμ for the excitation spectrum and an excitation wavelength of 360 mμ for the emission spectrum Control studies with known amounts of digoxin (0 1–0 5 μg) show elution from the thin layer plate to be about 76 %

Recovery studies from blood in vitro

Known amounts (10,20 and 30 μg) of digitoxin and digoxin, respectively are added to 30 ml of whole blood After standing for about 30 min at room temperature determination is performed according to the colorimetric method The recoveries of digitoxin for the three concentrations are respectively 70 55 and 40 % and for digoxin 100 95 and 90 %

Known amounts of digoxin (0 5 and 1 0 μg) are added to 10 ml of water and 10 ml of whole blood respectively After standing for about 30 min at room temperature

Table 1

Concentrations of digitalis glycoside in different organs in case 1 using the colorimetric method

	Conc of digitalis glycoside calculated as digitoxin μg/g (ml)	Total amount of digitalis glycoside calculated as digitoxin μg
Gastric contents	4 0	22
Contents of small intestine	1 3	38
Blood	0 7	
Heart	0 3	126

Table 2.

Concentrations of digoxin in different organs in case 2, using the colorimetric method

	Conc of digoxin µg/g	Total amount of digoxin µg
Heart	0 1	5
Liver	0 3	50
Lung	0 1	8

determination is performed by the fluorometric method The recovery from water is 74 % and from blood 47 and 41 % respectively

Case histories and findings

Case 1 A man, aged 40 years, weight 54 kg, swallowed for suicidal purposes about 50 digitalis tablets, each containing 50 mg standardized digitalis leaf, digitalis purpurea (0 5 i u) Death occured about 8 hours after the intake Using the colorimetric method described above the concentrations of digitalis glycoside found are shown in table 1

For the blood and the contents of the small intestine quantitative determination of digitoxin and gitoxin has been undertaken separately after thin layer chromatographic separation About one third of the total glycoside content represents gitoxin and about two thirds digitoxin The two glycosides have not been determined separately in the gastric contents and the heart

Case 2 A child was born with a congenital heart disease In hospital the child was treated with 0 1 mg digoxin intravenously three times a day over the last two days before death ½–1 hour before death the child was given in addition 0 7 mg digoxin intravenously Death occured when the child was 11 days old Weight 5 kg

Using the colorimetric method with xanthydrol the concentrations of digoxin determined are shown in table 2

It should be mentioned that the values obtained for the heart and lung are about at the limit of detection with the amount of organ available

Using the fluorometric method the concentrations of digoxin found are shown in table 3

Table 3

Concentrations of digoxin in different organs in case 2 using the fluorometric method

	Conc of digoxin µg/g (ml)	Total amount of digoxin µg
Heart	0 20	10
Liver	0.23	38
Kidney	0 68	37
Urine	0 38	0 6
Blood	0 15	

Discussion

The results of the chemical analyses in the two cases are in agreement with the view that digitalis poisoning was the cause of death

In 1969 ALHA & LUKKARI described 7 cases of digitalis poisoning in all of which a quantitative determination of digitalis glycosides had been performed with xanthydrol The concentration of digitalis glycosides found in fatal cases were about 25–150 μg per 100 g organ i e of the same order as those in the 2 cases described here In two of these cases in which the glycoside content was determined both in the kidney and the liver and in case 2 it is seen that the concentration in the kidney is more than twice the concentration in the liver

By means of the fluorometric method JELLIFE & STEPHENSON (1969) determined the myocardial digoxin concentrations and found it to be 0 210 μg/g and 0 262 μg/g respectively in two patients dying with overt digitalis toxicity from digoxin

In comparison with these results DOHERTY *et al* (1967) determined tissue concentration of tritiated digoxin in a patient who received 1 0 mg digoxin intravenously 5 ½ hours before death Our results are in agreement with this finding namely that the highest organ concentration is found in the kidney The kidney concentration is 0 14 μg/g, as compared with the heart and the liver concentrations which were 0 05 μg/g and 0 03 μg/g respectively

Methods much more sensitive than those used in the two cases described here and by which it is possible to determine plasma glycoside concentrations at clinical dosage levels have been described in recent years

A method based on the radioimmunoassay principle is described by BUTLER & CHEN (1967) EVERED *et al* (1970) and SMITH *et al* (1969) for the determination of digoxin in plasma and serum and by OLIVER *et al* (1968) for the determination of digitoxin in the serum The method seems technically difficult for use in the forensic chemical departments where the analysis for digitalis glycosides is performed only a few times a year

The method based on the inhibition of ^{86}Rb uptake by red cells is described by LOWENSTEIN (1965) LOWENSTEIN & CORRILL (1966) GRAHAME SMITH & EVEREST (1969) GJERDRUM (1970) BERTLER & REDFORS (1970) and BINNION *et al* (1969) for the determination of digoxin and digitoxin in plasma and serum BINNION *et al* (1969) also used the method for the determination of myocardial digoxin concentrations They determined both plasma and myocardial digoxin concentrations in patients who had been adequately digitalized according to clinical criteria and found the plasma concentrations to be in the range 0 1 2 5 mμg/ml with an average of 0 6 mμg/ml

The myocardial digoxin concentrations varied from 34 to 648 mμg/g with an average of 219 mμg/g

This method seems to be practical for forensic chemical analysis, providing a liquid scintillator is available

REFERENCES

Alha, A & I Lukkari Forensic chemical detection of digitalis glycosides *Progr Chem Tox* 1969 4 143–153

Bertler A & A Redfors An improved method of estimating digoxin in human plasma *Clin Pharmacol Therap* 1970 11, 665–673

Bićan Fišter, T & J Merkas Colorimetric assay of digitoxin and gitoxin in mixtures after thin layer chromatographic separation *J Chrom* 1969, 41, 91–95

Binnion P F, L M Morgan H M Stevenson & E Fletcher Plasma and myocardial digoxin concentrations in patients on oral therapy *Brit Heart J* 1969, 31, 636–640

Butler, V P & J P Chen Digoxin specific antibodies *Proc nat Acad Sci USA* 1967, 57, 71–78

Doherty J E W H Perkins & W J Flanigan The distribution and concentration of tritiated digoxin in human tissues *Ann Intern med* 1957, 66, 116–124

Evered D C C Chapman & C J Hayter Measurement of plasma digoxin concentration by radioimmunoassay *Brit Med J* 1970 3 427–428

Gjerdrum K Determination of digitalis in blood *Acta Med Scand* 1970 187 371–379

Grahame Smith D G & M S Everest Measurement of digoxin in plasma and its use in diagnosis of digoxin intoxication *Brit Med J* 1969, 1 286–289

Jelliffe R W A chemical determination of urinary digitoxin and digoxin in man *J Lab Clin Med* 1966 67, 694–708

Jelliffe R W & R G Stephenson A fluorometric determination of myocardial digoxin at autopsy with identification of digitalis leaf digitoxin and gitoxin *Amer J Clin Path* 1969, 51 347–357

Lowenstein, J M A method for measuring plasma levels of digitalis glycosides *Circulation* 1965, 31, 228–233

Lowenstein J M & E M Corrill An improved method for measuring plasma and tissue concentrations of digitalis glycosides *J Lab Clin Med* 1966, 67 1048–1052

Oliver G C, B M Parker, D L Brasfiels & C W Parker The measurement of digitoxin in human serum by radioimmunoassay *J Clin Invest* 1968, 47 1035 1042

Smith T W V P Butler & E Haber Determination of therapeutic and toxic serum digoxin concentrations by radioimmunoassay *New Eng J Med* 1969 281 1212 1216

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Distribution and Placental Transfer of ^{14}C-Thiourea and ^{14}C-Thiouracil in Mice Studied by Whole-body Autoradiography

By

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Abstract Whole body autoradiography of mice 5, 20 60, 240 min and 1 and 4 days after intravenous injection of ^{14}C-thiourea and ^{14}C-thiouracil showed a very similar distribution pattern for both substances The concentration of radioactivity in the thyroid was higher than the average concentration as early as 5 min after injection and it remained higher than in any other organ during the 4 days observation period A high radioactivity was also found in the walls of large blood vessels adrenal cortex and the mammary gland as well as in the liver lungs and the kidney where it persisted for as long as 24 hours In the foetuses a very high uptake of radioactivity was found in the thyroid while the concentration in the other foetal tissues as well as in placenta did not exceed that of the blood of mother

Key words Thiourea thiouracil – distribution – maternal foetal exchange

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The thiourea derivatives are widely used in the treatment of hyper thyroidism They inhibit the formation of thyroid hormone largely by interfering with the iodination of the tyrosyl groups (ASTWOOD 1949, MALOOF & SOODAK 1963) Several studies on their distribution and metabo lism in the body have been performed Using a chemical detection method WILLIAMS & KAY (1947) showed that thiourea and thiouracil are widely distributed in the tissues of the body and appear in the milk and in the urine In more detailed studies with ^{35}S labeled substances SCHULMAN (1950) and MALOOF & SOODAK (1957) found the highest concentrations of radio activity in the thyroid and kidney Several hours after the injection most of the radioactivity in the thyroid was present in the form of ^{35}S sulphate and some other ^{35}S labeled metabolites

Thiourea and thiouracil derivatives have also been shown to pass the placental barrier (HAYASHI & GILLING 1967) and to affect the offspring in

both animals and man (KAUFMAN *et al* 1948, PETERSON & YOUNG 1952, BURROW 1965, HERBST & SELENKOV 1965)

In the present investigation the distribution of ^{14}C-thiourea and ^{14}C-thiouracil has been studied with whole-body autoradiographic technique (ULLBERG 1954 & 1958) in order to elucidate further their fate in the organism and to see if the distribution is different from that of ^{35}S labeled compounds

Material and methods

^{14}C-thiourea (sp act 14 9 mci/mM) and 2 thiouracil 2 ^{14}C (sp act 8 6 mci/mM) were obtained from the Radiochemical Centre, Amersham, England The substances were dissolved in physiological saline and 0 2 ml was injected into a tail vein of each mouse

Adult white albino mice (NMRI) were used The male and female mice weighed 20–22 g and the pregnant mice (in a late state of gestation) weighed about 40 g All the mice were kept under the same conditions room temperature 25°, water *ad libitum* and they were fed a complete pelleted diet (AB Ewos Sweden)

Eight animals five male one non pregnant and two pregnant female mice received 10 μci ^{14}C thiourea (corresponding to about 0 05 mg) each The survival times were 5 min 20 min, 4 hours 1 day, and 4 days for the male mice 1 hour for the non pregnant female mouse and 20 min and 4 hours for the pregnant mice

Ten animals five male one non pregnant and four pregnant female mice received 5 μci 2 thiouracil 2-^{14}C (corresponding to 0 07 mg) each The survival periods for the male mice were 5 min 20 min 4 hours 1 day and 4 days for the non pregnant female mouse 1 hour and for the pregnant mice 20 min and 4 hours

After the survival periods stated above the mice were anaesthetized with ether and sacrificed by immersion into a mixture of hexane and solid CO_2 (−78°) Then the animals were embedded in a mixture of carboxymethyl cellulose and water Sagittal sections (20 μ) of the whole body were cut and dried at −15° according to ULLBERG s autoradiographic technique by which each section is attached onto tape (no 810 Minnesota Mining and Manufacturing Co (ULLBERG 1954 & 1958) The tape mounted sections were pressed against X ray films (Structurix D 7 Gevaert) and stored in a press at −15° After an exposure time varying from 2 weeks to 4 months, the films were developed and some of the sections were stained with haematoxylin and eosin

For semiquantitative evaluation of whole body autoradiograms the radioactivity in different organs were compared with the autoradiogram of a simultaneously exposed ^{14}C-isotope standard. Each standard consisted of 15 squares of different isotope concentration of ^{14}C arranged in a series of decreasing activity so that adjacent members of the series were related in the ratio ½ forming a staircase of radioactivity (BERLIN & ULLBERG 1963)

Results

The distribution pattern of ^{14}C thiourea and ^{14}C thiouracil were very similar Both substances were rapidly and evenly distributed in most tissues of the body The concentration of radioactivity in the thyroid was higher than the average concentration as early as 5 min after the injection In contrast to other tissues the radioactivity in the thyroid remained high

during the 4 days after injection, while it decreased in most tissues to very low level However, the liver, lung and kidney still showed appreciab amounts of radioactivity 24 hours after injection (fig 4)

Both substances were also found in the foetuses The foetal thyro showed a marked uptake, while the concentration in other foetal tissues well as in the placenta was low and uniform

The main route of excretion of both substances seemed to be via t kidney, but in addition there was some biliary excretion

The distribution and rate of uptake and disappearance in some tissu will be described in more detail below and the differences between ^{14}C-thiouracil and ^{14}C-thiourea will be pointed out

Blood and circulatory system

Both ^{14}C-thiourea and ^{14}C-thiouracil remained in detectable amounts the *blood* during the whole period of investigation After the injection ^{14}C-thiourea there was more radioactivity detectable in the blood 4 da after the injection than after the injection of ^{14}C-thiouracil Both substanc were accumulated for some time in the *arterial walls* (fig 1 & 2a)

The lymphatic organs

No specific uptake could be found in the *spleen, thymus* or in th *lymphatic glands*

The respiratory system

After the injection of ^{14}C-thiouracil or ^{14}C-thiourea the concentration the *lung parenchyma* was initially about the same as that in the blood, bu persisted for a longer time It exceeded the concentration in the bloo

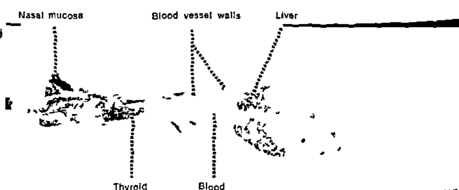


Fig 1 Autoradiogram of a male mouse 1 hour after intravenous injection of ^{14}C thiourea Note the accumulation (light areas) in the thyroid nasal mucosa, walls of the large vessels and in the liver

4 hours after the injection and remained higher for the remainder of the experiment (fig. 2a & 4)

The *nasal mucosa* and *bronchi* too showed a marked and persistent

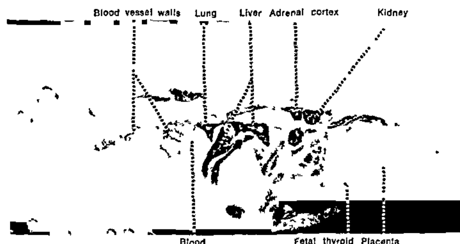
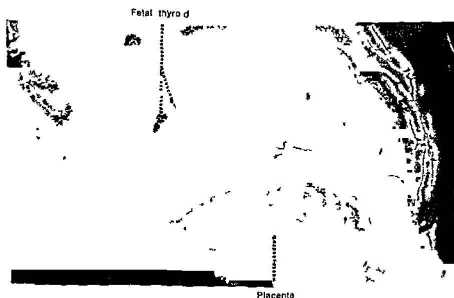


Fig 2a *Autoradiogram of a pregnant mouse 4 hours after intravenous injection of* ^{14}C-thiourea Note the high concentration of radioactivity (light areas) in the liver, lung, kidney, adrenal cortex, walls of large blood vessels and the foetal thyroid



2b Detail of the autoradiogram from fig 2a Note the selective uptake in the foetal thyroid

accumulation of the activity (fig 1), especially after the injection of ^{14}C-thiourea when a fairly high accumulation could be observed as long as 4 days after the injection

The digestive system

The *salivary glands* showed an uptake of both substances during the first hour after the injection The radioactivity then diminished rapidly and after 4 hours it did not exceed that of the blood More activity seemed to be localized in the mandibular gland than in the sublingual and parotid glands The *liver* had a high concentration of the activity as early as 5 min after the injection of either ^{14}C-thiouracil or ^{14}C-thiourea A high level persisted throughout the whole experiment (fig 2a & 4), and the activity could be detected as long as 4 days after the injection The radioactivity was apparently also excreted throughout the bile as a very high concentration could be detected in the bile and later on also in the intestinal contents The activity in the *stomach* and *intestinal mucosa* never exceeded that of the blood

The urinary system

The *kidney* already showed a high isotope concentration 5 min after the injection of ^{14}C-thiouracil and/or ^{14}C-thiourea The activity, which was mainly concentrated to the medulla and pelvis remained very high during the first hour and then decreased gradually (fig 2a & 4) After 4 days the concentration of the radioactivity was still higher than that of the liver The same was true for the *urinary bladder* and *ureter*, where the concentration of the radioactivity already reached the same level as that in the thyroid 5 min after the injection and persisted on that level for 4 hours

The reproductive system

In the male *sex glands* no specific uptake could be noted In the *ovaries* a moderate uptake of radioactivity was found in the corpora lutea

After the injection of either ^{14}C-thiourea or ^{14}C-thiouracil the *placenta* had a concentration corresponding to that of the maternal blood (fig 2a) The foetuses had generally a rather low and uniform activity A very high concentration could however, be observed in the foetal *thyroid* (fig 2a, 2b 3a & 3b) No uptake was found in the brain of the foetuses

The mammary glands of pregnant mice showed a marked accumulation of radioactivity after the injection of both ^{14}C thiourea and ^{14}C thiouracil (fig 3a)

The nervous system

No specific accumulation of radioactivity could be found in the CNS

The eye

The eye accumulated the radioactivity as early as 5 min after the injection of both ^{14}C-thiouracil and ^{14}C-thiourea The activity could be seen in the retina, iris and to some extent in the sclera The accumulation persisted for about 4 hours

The endocrine system

After the administration of either ^{14}C-thiouracil or ^{14}C thiourea the thyroid showed a rapid and marked uptake of the radioactivity, which persisted during the whole period of the investigation Five min after the injection the activity in the thyroid was about 4 times as high as in the blood and remained at that level up to 4 hours At that time only the kidney and the bladder contained activities of a similar magnitude After 1 day and 4 days, when the activity in the blood became quite low, the concentration in the thyroid decreased to only about one half (fig 1 & 4) The *parathyroid* did not accumulate any appreciable amount of the activity In the *adrenal cortex* there was a rapid uptake of activity with the concentration exceeding that of the blood for up to 4 hours when it started to decrease gradually (fig 2a) The concentration of the activity was more pronounced after the injection of ^{14}C thiourea and was concentrated mainly to the zona fasciculata Very little activity could be detected in the *adrenal medulla* after the injection of both ^{14}C-thiouracil and ^{14}C thiourea The same was true for the *islets of Langerhans*

Muscles

The skeletal muscles showed a moderate uptake 5 min after the injection but it decreased with time

The hard tissues

No ^{14}C was registered detected in the bone The cartilage and tendons accumulated some activity during the first hour after the injection

DISCUSSION

The most striking finding in the present work is the very marked uptake and retention of radioactivity in both the maternal and foetal thyroid glands In the foetus the uptake is almost selective in the thyroid with the remainder of the foetal tissues showing only low and uniform concentration of radioactivity

This may be related to the well known types of thyroid dysfunction and morphological changes which are frequently found in the newborn in both man and animals after medication with thyrostatic compounds of the

thiourea type during pregnancy (FRISLEBEN & KJERULF-JENSEN 1947, PETERSON & YOUNG 1952, AARON *et al* 1955, BURROW 1965) The most remarkable dysfunction finding is foetal goiter (BURROW 1965, HERBST & SELENKOW 1965) A possible mode of action in the development of this goiter is that the thiourea compound, which has entered the foetus, blocks the formation of iodinated thyroid hormones normally taking place in the foetal thyroid during late gestation (CHAPMAN *et al* 1948, HODGES *et al* 1955, GELOSO 1956), the consequent increased production of foetal thyrotropic hormone may thus induced an extensive growth of the foetal thyroid

Our results are in agreement with those of SHEPHARD (1963), who reported an uptake of ^{35}S-labeled thiouracil into the rat foetal thyroid using liquid scintillation counting. Placental transfer of labelled thiourea derivatives has been shown by HAYASHI & GILLING (1967), HAYASHI *et al* (1970) and SABBAGHA *et al* (1969), who measured the radioactivity in whole foetuses

The present finding of a selective accumulation of thyrostatic agents in the foetal thyroid is one of many examples of selective accumulation of drugs, which cause foetal damage in the critical foetal organ Other examples are selective accumulation of tetracyclines in the foetal skeleton (BLOMQUIST & HANNGREN 1966) and of chloroquine and chlorpromazine in the melanin of the foetal eye (ULLBERG *et al* 1970)

The very prolonged retention of radioactivity in the thyroid is probably

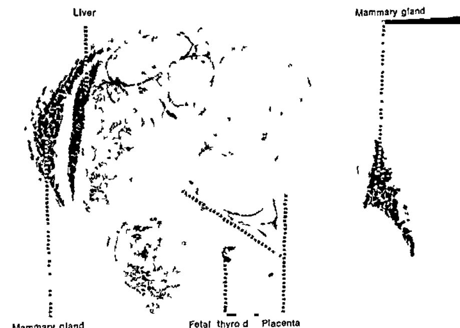


Fig 3a

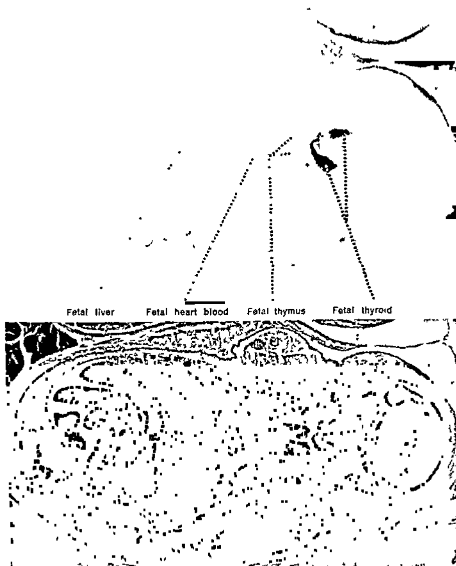


Fig 3b

Fig 3a Autoradiogram of a pregnant mouse 4 hours after intravenous injection of ^{14}C-thiouracil Note the high accumulation (light areas) in the foetal thyroid and in the mammary gland of the mother The activity is also present in the liver of the mother 3b Detail of the autoradiogram from fig 3a and corresponding stained section (lower) Note the selective accumulation of radioactivity (light areas) in the foetal thyroid

4 Autoradiogram of a male mouse 24 hours after intravenous inject on of : thiouracil Note the very high concentration of rad oactivity (light areas) in the thyro d Activity is also present in the lung liver and kidney

e to the participation of ^{14}C-containing breakdown products in the /roid metabolism In relation to this it can be mentioned that MALOOR & ODAK (1957) found the radioactivity of ^{35}S thiourea after 6 hours to present only about two percent of thiourea whereas the major part (of the lioactivity) was present in the form of sulphate The metabolism of ^{35}S ouracil however seems to proceed somewhat more slowly

In contrast to the foetus the maternal tissues showed many other sites of alization It was mainly at late times after the injection (24 hours and lays) that the thyroid totally dominated the maternal distribution picture time when the radioactive compounds had largely left the remaining ues)

The retention of radioactivity in the lung and the nasal mucosa may related to the presence of a special odour in the breath described by ier investigators after the administration of thiourea (VAN DER LAAN & RRIE 1955)

The adrenals are also known to undergo a considerable atrophy and lfunction as a result of treatment with thiourea compounds treatment vever the same changes can be reproduced by thyroidectomy and vented by simultaneous injection of thyroid hormone with the antithyroid stance (Cit f GREER *et al* 1964) In view of the high accumulation of ioactivity observed in this structure it is possible that these drugs have least a partial direct toxic effect on the adrenals

An interesting finding is the high uptake of label by the walls of elastic eries A similar distribution pattern has also been observed with an ionic roid inhibitor thiocyanate (CLEMEDSON *et al* 1960) and with iod ne LLBERG & EWALDSSON 1964)

A marked accumulation of radioactivity in the mammary glands of

pregnant mice indicates that the thiourea compounds are excreted with the milk, thus representing another risk for the nursing infants

Similarly an uptake and retention of both ^{14}C thiourea and ^{14}C-thiouracil and/or their metabolites in the kidney and the urinary system reflect the main secretory pathway for both compounds, which is in agreement with previous findings (WILLIAMS & KAY 1947, SCHULMAN & KEATING 1950, ALEXANDER *et al* 1969)

REFERENCES

Aaron H H S J Schneierson & E Siegel Goiter in newborn infant due to mothers ingestion of propylthiouracil *JAMA* 1955 **159** 848–850

Alexander W D V Evans A MacAulay T F Gallagher & J Londono Metabolism of ^{35}S-labelled antithyroid drugs in man *Brit Med J* 1969 **2** 290–291

Astwood E B Mechanisms of action of various antithyroid compounds. *Ann N Y Acad Sci* 1949 **50** 419–443

Berlin M & S Ullberg Accumulation and retention of mercury in the mouse *Arch Env Hlth* 1963 **6** 589–601

Blomquist L & A Hanngren Fluorescence technique applied to whole body sections for distribution studies of tetracyclines *Biochem Pharmacol* 1966 **15** 215–219

Burrow G N Neonatal goiter after maternal propylthiouracil therapy *J clin Endocrin* 1965 **25** 403–408

Chapman E M G W Corner D Robinson & R D Evans The collection of radioactive iodine by the human fetal thyroid *J clin Endocrin* 1948 **8** 717–720

Clemedson C J B Sorbo & S Ullberg Autoradiographic observations on injected S^{35} thiocyanate and C^{14} cyanide in mice *Acta physiol scand* 1960 **48** 382–389

Freisleben R & K Kjerulf Jensen Effect of thiouracil derivatives on fetuses and infants *J clin Endocrin* 1947 **7** 47–51

Geloso J P Recherches sur le metabolisme de l iode radioactif par la thyroide du foetus de rat *C R Soc Biol* (Paris) 1956 **150** 2140–2143

Greer M A J W Kendall & M Smith In *The thyroid gland* Ed Pitt Rivers and W R Trotter Butterworths London 1964 p 363

Hayashi T T & B Gilling Placental transfer of thiouracil *Obstet Gynec* 1967 **30** 736–740

Hayashi T J Teubner & B Gilling Study of placental transfer of thiouracil derivatives *Amer J Obstet Gynec* 1970 **108** 723–728

Herbst A & A Selenkov Hyperthyroidism during pregnancy *New Engl J Med* 1965 **273** 627–633

Hodges R E T C Evans J T Bradbury & W C Keettel The accumulation of radioactive iodine by human fetal thyroids *J clin Endocrin* 1955 **15** 661–667

Kaufman G V V Hurst & C W Turner Histological effect of thiouracil on the fetal thyroid of the mouse *Endocrinology* 1948 **43** 187 191

Maloof F & M Soodak The uptake and metabolism of S^{35} thiourea and thiouracil by the thyroid and other tissues *Endocrinology* 1957 **61** 555 569

Maloof F & M Soodak Intermediary metabolism of thyroid tissue and the action of drugs *Pharmacol Rev* 1963 **15** 43 95

Peterson R R & W C Young The problem of placental permeability for thyrotrophin propylthiouracil and thyroxine in the guinea pig *Endocrinology* 1952 **50** 218–225

Sabbagha R E & T T Hayashi Transfer of ^{14}C-thiouracil during pregnancy *Amer J Obstet Gynec* 1969, 103, 121–127

Schulman J Studies on the metabolism of thiourea II The metabolic fate of thiourea in the thyroid gland *J Biol Chem* 1950 186 717–723

Schulman J & R P Keating Studies on the metabolism of thiourea I Distribution and excretion in the rat of thiourea labeled with radioactive sulfur *J Biol Chem* 1950, 183 215–221

Shephard T H Metabolism of thiourea S^{35} by the fetal thyroid of the rat *Endocrinology* 1963, 72 223–231

Ullberg S Studies on the distribution and fate of S^{35}-labelled benzylpenicillin in the body *Acta radiol* (Stockholm) 1954 Suppl 118, 1–110

Ullberg S Autoradiographic studies on the distribution of labelled drugs in the body *Second U N Conf Peaceful Uses of Atomic Energy* 1958, 24, 248–254

Ullberg, S & B Ewaldsson Distribution of radio-iodine studied by whole-body autoradiography *Acta radiol* 1964, 2, 24–32

Ullberg S, N G Lindquist & S E. Sjostrand Accumulation of chorio-retinotoxic drugs in the foetal eye *Nature* 1970, 227, 1257–1258

van der Laan W P & M Storrie A survey of the factors controlling thyroid function with especial reference to newer views on antithyroid substances *Pharmacol Rev* 1955, 7, 301–334

Williams, R. H & G A Kay Thiouracils and thioureas Comparisons of the absorption, distribution destruction and excretion *Arch Int Med* 1947, 80, 37–52

Acta pharmacol et toxicol 1973, 32, 369–381.

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The Effect of Guanethidine Pretreatment on Transmission in the Superior Cervical Ganglion

By

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Abstract Isolated superior cervical ganglia from rats pretreated with guanethidine sulphate intraperitoneally in single daily doses of 20–40 mg/kg for 5–21 days showed transmitted action potentials of normal form but diminished amplitude following single or repetitive stimulation of the preganglionic nerve Curves relating the relative reduction in ganglionic action potential height with increasing frequency of stimulation were similar in preparations from control and guanethidine pretreated rats Ganglia from guanethidine pretreated rats had the same sensitivity to exogenous acetylcholine as those from the controls as judged by dose/depolarization response curves The absolute values of depolarization were, however, diminished No impairment of the conduction of impulses in the preganglionic nerve or of the effect of antidromic stimulation was observed The addition of guanethidine to the bath fluid resulted in an acute reversible ganglion block A 50 % reduction of the ganglionic action potentials following single stimuli was observed at a concentration of 2×10^{-5} M At guanethidine concentrations of 5×10^{-5} M maximum depolarizations obtained with acetylcholine or carbachol were reduced by approximately 50 % but the dose/response curves were not shifted Recovery was complete after washing Retractions of the cat nictitating membrane following repetitive stimulation of the preganglionic nerve after pretreatment with guanethidine 5–40 mg/kg for 14 days were preferentially decreased at low stimulus frequencies The nictitating membrane was hypersensitive to intra venous adrenaline or noradrenaline Contralateral ganglia from both rats and cats exhibited profound structural changes consisting of chromatolysis of the nerve cells and infiltration of small cells The effects of guanethidine on the neurophysiological parameters are probably the result of a dose dependent inactivation of the majority of the ganglion cells

Key words Guanethidine – ganglionic transmission rat – cat

Prolonged administration of guanethidine sulphate in the rat at doses above 10 mg/kg daily caused enlargement of the superior cervical ganglion together wit

activity, partial depletion of catecholamines, structural changes consisting of chromatolysis of the nerve cells accompanied by an infiltration of small cells, and ultrastructural changes in the nerve cells dominated by mitochondrial swelling (JENSEN-HOLM 1967, JENSEN HOLM & JUUL 1970a b & 1971) This was not seen with lower doses (BURNSTOCK *et al* 1971a)

An acute but transient blocking action of guanethidine in cat sympathetic ganglia has been recognized but does not seem to be responsible for the long term action of guanethidine (MAXWELL *et al* 1960, BEIN 1960 GERTNER & ROMANO 1961, KADZIELAWA & GUMULKA 1967)

The present experiments were carried out to see whether the histological changes induced by guanethidine were reflected in changes in ganglionic function The acute effects of guanethidine added to the preparation *in vitro* were also studied Finally the response of the nictitating membrane to stimulation of the superior cervical preganglionic nerve was studied in cats pretreated with guanethidine The histology of the contralateral ganglia was also investigated

Material and methods

Wistar strain rats of either sex as stated in Results weighing 160–200 g received intraperitoneally either guanethidine sulphate (ismelin® CIBA 10 mg/ml dissolved in saline) in single daily doses of 20 or 40 mg/kg or 0 5 ml of normal saline for 5 to 21 days All the animals treated with guanethidine exhibited ptosis after 24 hours One or two days after the last injection the rats were anaesthetized with 1 0–1 5 g/kg of 25 % w/v urethane intraperitoneally the right superior cervical ganglion was excised together with 1 2 cm of the preganglionic sympathetic trunk and 3–4 mm of the postganglionic internal carotid nerve immersed in Krebs solution equilibrated with 95 % O_2 and 5 % CO_2 at room temperature and the connective tissue sheath removed

The electrical activity of the preparations following single or repetitive supra maximal stimuli to the preganglionic trunk (fig 1) was recorded using a slight

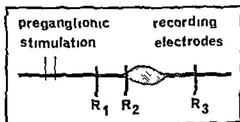


Fig 1 Position of stimulating and recording electrodes (R_1 R_2 and R_3) on the isolated rat superior cervical ganglion The distance between R_1 and R_2 was approximately 2 mm

modification of the external recording technique of QUILLIAM & SHAND (1964) Action potentials following single maximal preganglionic stimuli of 0 5 msec duration were recorded 6 times at 5 min intervals and following repetitive stimulation for 10 sec at rates of 1 2 4 6, 8, and 10 Hz with a 15 min rest period between each train

Depolarization of the ganglia by added ACh or carbachol was measured by the moving fluid electrode technique of PASCOE (1956) The depolarizing agent was added for 4 min at not less than 30 min intervals

Cats of either sex weighing 2 6–4 0 kg received guanethidine sulphate intraperitoneally in single daily doses of 5, 10 20, or 40 mg/kg for 14 days The cats used for the experiments in fig 6 and 7 were from the same litter The control animals received no injections All animals after treatment with guanethidine for 24 hours exhibited relaxation of the nictitating membrane 24 hours after the final injection the animals were anaesthetized with chloralose 50 mg/kg w/v and urethane 500 mg/kg w/v intraperitoneally Supramaximal rectangular shocks of 0 5 msec. duration at each of a series of increasing frequencies from 1 to 30 Hz for 1 min periods followed by 2 min rest periods were applied to the preganglionic nerve trunk by means of a Multistim DISA stimulator The isotonic contraction of the nictitating membrane was measured by a displacement transducer (Hewlett Packard load 4 7 g) coupled to a Beckman Dynograph S II at 5 times magnification

The drugs used were guanethidine sulphate (ismelin®, CIBA) clonidine hydrochloride (catapres® BOEHRINGER) adrenaline and noradrenaline bitartrate, acetyl choline chloride and carbachol chloride

The contralateral ganglia in both the rat and cat experiments were excised and used for histological examination as described by JENSEN HOLM & JUUL (1970b) using the gallocyanine chromalun method of EINARSON (1932) for the demonstration of the Nissl substance the silver impregnation technique of BODIAN (1936) for the demonstration of nerve fibres or a combination of these two methods

The statistical calculations are based on Student s t test

Results

Rat experiments

a) Transmission studies Initial studies were undertaken in 5 female rats, each receiving guanethidine in different dose regimens as follows 20 mg/kg for 10 and 12 days 40 mg/kg for 5 and 10 days, and 20 mg/kg for 12 days followed by 40 mg/kg for 5 days The transmitted action potentials in control ganglia have a typical shape exhibiting Sa N and P waves as seen in fig 2 and 3 All preparations from the guanethidine pretreated rats in response to single preganglionic stimuli showed transmitted ganglionic action potentials of normal form but of a dose dependent diminished amplitude – the potentials being approximately 50 % of the control value following pretreatment with 40 mg/kg for 10 days A typical recording is shown in fig 2B The investigation was extended using 6 male rats 3 following pretreatment with guanethidine sulphate 20 mg/kg for 21 days and 3 following 40 mg/kg for 21 days, thus allowing a statistical evaluation of the results The voltage necessary to obtain supramaximal preganglionic

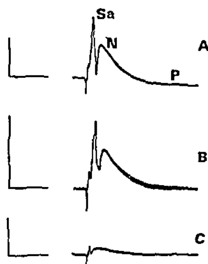


Fig 2 Action potentials recorded from rat isolated superior cervical ganglia following single supramaximal stimuli to the preganglionic nerve trunk Position of the recording electrodes R_2R_3 (fig 1)

A Untreated ganglion

B Ganglion from rat pretreated with guanethidine sulphate 20 mg/kg for 12 days followed by 40 mg/kg for 5 days

C. As in A but after the addition to the bath fluid of guanethidine 3.4×10^{4} M Calibrations 200 msec and 4 mV The different magnification in B should be noted.

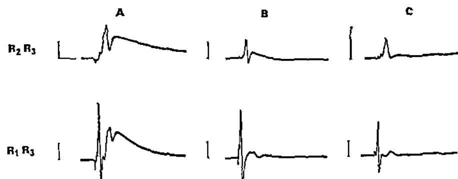


Fig 3 Action potentials recorded from rat isolated superior cervical ganglia following single supramaximal stimulation of the preganglionic nerve trunk Position of the recording electrodes see fig 1

A Untreated rat

B Rat pretreated with guanethidine sulphate 20 mg/kg for 21 days

C Rat pretreated with guanethidine sulphate 40 mg/kg for 21 days

Calibrations 50 msec and 1 mV The different magnification in C (R_2R_3) should be noted

Table I

Size in mV of potentials recorded from isolated rat superior cervical ganglia following *single* and *repetitive supramaximal stimulation* of the preganglionic nerve *trunk and* maximal acetylcholine (ACh) *depolarization* obtained at *17.6 mM* ACh in the bath fluid. Sa and N ganglionic potentials recorded with the recording electrodes in *position* R_2-R_3 (fig 1) *pre preganglionic nerve action potential recorded* with *recording electrodes* in position R_1 R_3 (fig 1) *n = number of animals. Mean values* and S.E.M. (in brackets) Open circles $P < 0.05$, *filled* circles $P < 0.01$ asterisks $P < 0.001$ (t test)

Drug and dose mg/kg	Days	Single stimulus *n*	Sa	N	*n*	pre	Repetitive stimulation Sa n	1 Hz	10 Hz	Depol ACh n	17.6 *mM*
Controls		10	3.5 (0.3)	1.4 (0.1)	6	2.6 (0.4)	8	4.1 (0.3)	1.2 (0.2)	4	3.4 (0.3)
Guanethidine sulphate 20	21	3	1.0 (0.3)★	0.3 (0.1)●	3	2.7 (0.4)	3	1.1 (0.4)★	0.3 (0.2)○	3	1.2 (0.3)●
Guanethidine sulphate 40	21	3	0.6 (0.2)★	0.2 (0.2)★	3	3.1 (0.5)	3	0.7 (0.3)★	0.2 (0.1)●	3	0.7 (0.2)★

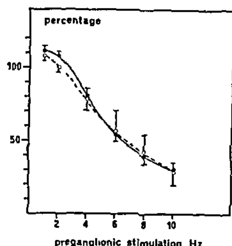


Fig 4 Comparison of mean gangl on action potentials (Sa) recorded at various frequencies of supramaximal stimulation of the preganglionic nerve trunk of rat isolated superior cervical ganglia
Abscissa Frequency of stimulation
Ordinate 5th action potential expressed as per cent of the first response (mean ± S.E M)
Full line Control ganglia (n = 8)
Broken line Ganglia from rats pretreated with guanethidine sulphate 20 and 40 mg/kg for 21 days (n=6)

stimulation was identical in the control experiments and in those following guanethidine pretreatment (range 2-20 V in both groups) The results are given in table 1, and representative recordings are illustrated in fig 3 A statistically significant reduction in the height of the Sa and N waves followed guanethidine pretreatment The P wave was too small (approximately 0 2 mV) to allow assessment of differences between control and guanethidine pretreated ganglia No impairment of the conduction of impulses in the preganglionic nerve was observed

The responses at each frequency of repetitive stimulation may be plotted as the percentage of the first response at 1 Hz. The graphs in fig 4 plotted in this way show that the mean responses are similar for the control and guanethidine pretreated rats Data from rats pretreated with guanethidine sulphate 20 or 40 mg/kg were cumulated since the results show only minor variations without dose dependence The absolute values following guanethidine pretreatment are lower at all frequencies of stimulation the mean reduction with 20 or 40 mg/kg for 21 days being 75 and 81 % respectively In table 1 the figures at 1 and 10 Hz are shown

The addition of guanethidine to the bath fluid resulted in a reversible ganglion block in the isolated ganglion preparation (fig 2C) The Sa potential with single stimuli was reduced by 10 % at a guanethidine concentration of $3\,4 \times 10^{-6}$ M, by 50 % at 2×10^{-5} M and by 96 % at $5\,5 \times 10^{-4}$ M The response to repetitive stimulation when plotted as in fig 4 was depressed by guanethidine $1\,4 \times 10^{-5}$ M at low frequencies of stimulation (at 1 Hz $P < 0\,02$), at higher frequencies there was no significant change The three concentrations were without effect on the preganglionic or antidromic action potential heights

b) Depolarization studies The ACh dose/mean peak depolarization

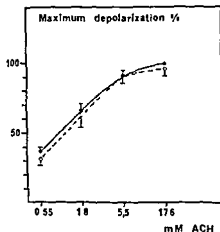


Fig 5 Acetylcholine (ACh) depolarization curves from rat isolated superior cervical ganglia Maximum depolarization at 18 mM ACh was taken as 100 % and the other values are expressed as per cent of this value (mean ± S E M)
Full line Control ganglia (n = 4)
Broken line Ganglia from rats pretreated with guanethidine sulphate 20 and 40 mg/kg for 21 days (n=6)

voltage response curves from control ganglia and guanethidine pretreated ganglia were almost superimposable (fig 5), whereas the absolute values were depressed (table 1) The time course of the depolarization and post washing hyperpolarization likewise did not differ between control and guanethidine pretreated ganglia In a few experiments with carbachol ($5\ 5 \times 10^{-6} - 5\ 5 \times 10^{-4}$ M), the results mimicked those with ACh

In a few experiments guanethidine in concentrations of 3 4 to $6\ 8 \times 10^{-5}$ M was added to the fluid bathing an isolated ganglion preparation The peak depolarizations of control ganglia with each dose of ACh or carbachol were reduced by approximately 50 % but the dose/response curves were not shifted

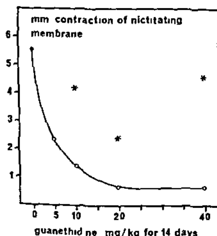


Fig 6 Isotonic retraction in mm of cat nictitating membrane following supra maximal stimulation of the preganglionic nerve trunk Control cat (solid point) and cats pretreated with guanethidine sulphate at various dose levels The asterisks indicate the maximal contraction elicited by adrenaline 33 μg/kg intravenously

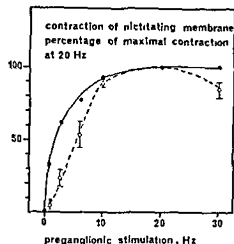


Fig 7 Contraction of cat nictitating membrane following supramaximal stimulation at various rates of the preganglionic nerve trunk The contraction at 20 Hz was taken as 100 % and all other values expressed as per cent of this value (mean ± S E M)
Full line Control cats (n = 2)
Broken line Response from guanethidine sulphate pretreated cats at doses from 5 to 40 mg/kg for 14 days (n=4)

Cat nictitating membrane

The response of the nictitating membrane to supramaximal stimulation of the preganglionic nerve was reduced in cats pretreated with guanethidine (fig. 6) although the membrane retracted strongly to intravenous adrenaline The maximal adrenaline induced contractions of the nictitating membrane in 4 control cats were 4 7–6 0 mm, in 5 guanethidine pretreated cats 2 4–5 5 mm The response of the nictitating membrane was preferentially reduced at low repetitive stimulation rates (fig 7) The voltage necessary to obtain supramaximal preganglionic stimulation was 4–9 V in 3 control cats and 1–8 V in 7 guanethidine pretreated cats

The nictitating membranes in cats pretreated with guanethidine responded to 0 2 µg/kg adrenaline and noradrenaline injected intravenously whereas much higher doses (more than 10 µg/kg) were required to induce contractions in the control cats Similarly nictitating membranes of guanethidine pretreated cats responded to small doses of guanethidine (1 mg/kg) and clonidine (0 5 µg/kg) intravenously whereas in the control cats more than 2 mg/kg and 9 µg/kg respectively were required to give comparable responses

Histological studies

Both rat and cat ganglia showed histological alterations following pretreatment of the animals with guanethidine consisting of chromatolysis of the nerve cells accompanied by infiltration of small cells (fig 8B and D) Cell counts and the ratio of small cells to ganglion cells in the rat experiments are given in table 2 At 20 and 40 mg/kg almost all the ganglionic nerve cells showed severe chromatolytic changes

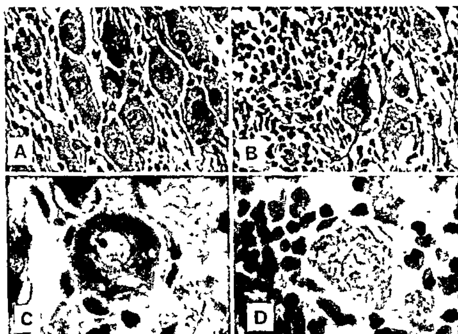


Fig. 8 Histological appearance of superior cervical ganglion. A and B stained by a combined silver impregnation – gallocyanine chromalun method, C and D stained only by the gallocyanine chromalun method.
A Control cat, B Cat pretreated with guanethidine sulphate 20 mg/kg for 14 days. Magnification × 200.
C Control rat, D Rat pretreated with guanethidine sulphate 20 mg/kg for 21 days. Magnification × 500.

Table 2

Cell counts in histological specimens of rat superior cervical ganglia from control rats and from rats pretreated with guanethidine at two dose levels for 3 weeks. In the controls, cells within an area of 0.1 mm² per specimen were counted, in guanethidine pretreated ganglia, cells within an area of 0.3 mm² were counted. n = number of rats. Mean values and S.E.M. (in brackets). Open circles: P < 0.05 (t test). * Data from JUUL & SAND (1973).

Drug and dose mg/kg	Days	n	ganglion cells per mm²	small cells per mm²	small cells / gangl. cells
Controls*		28	696 (26)	3448 (108)	5.0 (0.2)
Guanethidine sulphate 20	21	3	278 (68)○	9141 (1141)○	40.3 (15.4)
Guanethidine sulphate 40	21	3	344 (34)○	9363 (1203)○	28.6 (6.9)

The absolute number of ganglion cells per ganglion was not counted Irreversible degeneration of 25–30 % of the ganglionic nerve cells was estimated following guanethidine pretreatment at the dosages used since the number of ganglion cells counted per mm² was approximately halved (table 2) whereas the size of the ganglia has been shown to increase by approximately 60 % (JUUL & SAND 1973)

In the cat experiments similar changes were observed, minor changes occurring at 10 mg/kg, maximal changes at 20 mg/kg for 14 days There were considerable variations between the specimens not related to the dose but no loss of ganglion cells was observed

There was no correlation between the severity of the histological changes and the parameters investigated electro-physiologically in the individual rats and cats

Discussion

The present findings of *ganglionic potentials* of normal form, although of diminished amplitude were unexpected because of the very profound biochemical and structural changes seen in the superior cervical ganglion following prolonged administration of guanethidine (JENSEN HOLM & JUUL 1970a, b & 1971, BURNSTOCK *et al* 1971b) Similar findings of apparently normal postganglionic action potentials following prolonged administration of large doses of guanethidine in the cat have been reported by MAXWELL *et al* (1960) and by BEIN (1960)

The presence of these ganglionic action potentials suggests that some at least, of the ganglionic synapses are transmitting The plot of the pooled responses to repetitive stimulation in fig 4 offers support for the view that the ganglionic transmission is qualitatively unimpaired It should be noted that the majority of the nerve cells in ganglia from rats pretreated with large doses of guanethidine displayed profound histological changes The actual number of completely degenerating nerve cells however, was only 25–30 % and a chemical sympathectomy as described by BURNSTOCK *et al* (1971b) following 25–30 mg/kg for 6 weeks was not observed in the present investigation It seems likely that some of the ganglion cells are more susceptible to the damaging effect of guanethidine than others, the inactivation of the majority of the cells resulting in reduced ganglionic action potentials Several investigators have suggested that the sympathetic ganglia contain two or more different types of nerve cells e g KLINGMAN & KLINGMAN (1965) in a study of the effect of immunosympathectomy They demonstrated a loose correlation between the number of surviving nerve cells and the magnitude of the action potential They could not state, however, whether or not the action potentials recorded from anti serum

treated rats were of the same nature, only reduced as those recorded from control ganglia A similar conclusion applies to our present study

Postganglionic axotomy induces histological cholinesterase and catecholamine changes bearing resemblance to those following guanethidine administration (JENSEN HOLM & JUUL 1970a b & 1971) BROWN & PASCOE (1954) found a complete failure of transmission in excised ganglia following postganglionic axotomy By examination *in vivo* however, small remnants of transmitted responses were observed A prolongation of the relative refractory period manifested itself by the failure of the ganglion to transmit repetitive impulses and in this respect the results in guanethidine pretreated animals are different The temporal course of the morphological changes following guanethidine accords with the electro physiological findings after postganglionic axotomy (ACHESON & REMOLINA 1955)

The results of the experiments in the *cat nictitating membrane* following pretreatment with guanethidine confirm the results of BOURA & GREEN (1962) both in general and in detail the preferential impairment of the responses at low stimulation frequencies (fig 7) and to the hypersensitivity to catecholamines and guanethidine injected intravenously A similar hypersensitivity was found to the newer drug clonidine injected intravenously BOURA & GREEN (1962) reported a complete block of response to nerve stimulation in 4 cats given 10 mg/kg of guanethidine sulphate daily for 14 days In the present investigation 3 cats receiving 10 20 and 40 mg/kg for 14 days responded weakly to nerve stimulation (fig 6) The nictitating membranes of guanethidine pretreated cats are supersensitive to catecholamines but apparently respond with a normal contraction height to stimulation by catecholamines injected intravenously indicating that the diminished contraction following preganglionic stimulation is not due to guanethidine induced changes at the end organ

The *histological changes* in the cat following guanethidine were identical to those described in the rat (JENSEN & JUUL 1970b) The changes of the neurophysiological parameters investigated showed dose dependence in both rat and cat experiments However no correlation between the severity of the histological changes and the changes in ganglionic potential heights the magnitude of the acetylcholine depolarizations and the heights of the contractions of the nictitating membrane could be established

The *in vitro experiments* in which guanethidine was added to the fluid bathing an isolated ganglion have confirmed the findings of others of a reversible ganglionic blocking activity of guanethidine (MAXWELL *et al* 1960 BEIN 1960 GERTNER & ROMANO 1961 KADZIELAWA & GUMULKA 1967) GERTNER & ROMANO (1961) demonstrated a complete block by guanethidine when the cat superior cervical ganglion was perfused at a concentration of 4×10^{-5} M This concentration is close to the concen

tration *in vitro* which blocked ganglionic transmission in the present investigation Although it has been shown that guanethidine cumulates in sympathetic ganglia (JUUL & SAND 1973) the concentration at the ganglionic synapses is unknown, and the present study shows that the ganglionic block induced by guanethidine *in vitro* appears to be different from the impairment of ganglionic function observed following prolonged administration, since the latter is not reversed by washing for several hours

The normal relative sensitivity to acetylcholine found in the *depolarization studies* (fig 5) is striking in the light of the pronounced depression of the cholinesterase activities described by JENSEN-HOLM & JUUL (1970a & b) The greater part of the loss of enzyme, however, was localized to the ganglion cell cytoplasm whereas the activity of the preganglionic nerve fibres appeared to be normal It should be noted that although the maximal response to ACh was reduced in both ganglia from guanethidine pretreated rats and *in vitro* experiments the dose/response curve to ACh did not shift to the left It has been suggested that the drug responses obtained with the fluid electrode technique are directly related to the number and integrity of the neurones (WATSON 1970)

Several factors make it difficult to draw any conclusions as to the importance of the findings of reduced amplitudes of the ganglionic potentials following preganglionic stimulation in guanethidine pretreated rats Technical differences between the individual experiments (e g the position of the stimulating and recording electrodes) and the increased size of the ganglia in the guanethidine pretreated rats may interfere to an unknown degree with the exact comparison of the potentials It seems reasonable to assume, however, that the present findings are the result of a decreased number of functioning neurones caused by a damaging effect of guanethidine, specifically cumulating in the sympathetic postganglionic neurones The mechanism underlying this effect of guanethidine remains to be clarified, and it should be emphasized that the changes observed in this preparation and under the experimental conditions used do not allow any extrapolation to the suggestion of a mechanism of action of guanethidine at therapeutic doses during longterm administration

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REFERENCES

Acheson, G H & J Remolina The temporal course of the effects of postganglionic axotomy on the inferior mesenteric ganglion of the cat *J Physiol* (London) 1955, 127, 603–616

Bein, H J Some pharmacological properties of guanethidine In *Adrenergic mechanisms* CIBA Foundation Churchill, London 1960, 162–172

Bodian, D A new method for staining nerve fibers and nerve endings in mounted paraffin sections *Anat Rec* 1935 65, 89–97

Boura, A L A & A F Green Comparison of bretylium and guanethidine Tolerance, and effects on adrenergic nerve function and responses to sympathomimetic amines *Brit J Pharmacol* 1962, 19, 13–41

Brown, G L & J E Pascoe The effect of degenerative section of ganglionic axons on transmission through the ganglion *J Physiol* (London) 1954, 123, 565–573

Burnstock, G, A E Doyle, B J Gannon, J F Gerkens, T Iwayama & M L Mashford Prolonged hypotension and ultrastructural changes in sympathetic neurones following guanacline treatment *Eur J Pharmacol* 1971a, 13 175–187

Burnstock, G, B Evans, B J Gannon J W Heath & V James A new method of destroying adrenergic nerves in adult animals using guanethidine *Brit J Pharmacol* 1971b, 43, 295–301

Einarson, L A method for progressive selective staining of Nissl and nuclear substance in nerve cells *Amer J Path* 1932, 8, 295–308

Gertner, S B & A Romano Action of guanethidine and bretylium on ganglionic transmission *Fed Proc* 1961, 20, 319

Jensen Holm, J Cholinesterase and protein changes in sympathetic ganglia after guanethidine *Acta pharmacol et toxicol* 1967, 25, suppl 4 74

Jensen Holm, J & P Juul The effects of guanethidine, pre and postganglionic nerve division on the rat superior cervical ganglion Cholinesterases and protein *Acta pharmacol et toxicol* 1970a, 28 270–282

Jensen Holm, J & P Juul The effects of guanethidine pre and postganglionic nerve division on the rat superior cervical ganglion Cholinesterases and catecholamines (histochemistry) and histology *Acta pharmacol et toxicol* 1970b, 28 283–298

Jensen Holm, J & P Juul Ultrastructural changes in the rat superior cervical ganglion following prolonged guanethidine administration *Acta pharmacol et toxicol* 1971 30, 308–320

Juul P & O Sand Guanethidine determination in sympathetic ganglia *Acta pharmacol et toxicol* 1973 33, *in press*

Kadzielawa, K & W Gumulka Influence of new guanidine derivatives on ganglionic and neuromuscular transmission *Arch int Pharmacodyn* 1967 170, 297–309

Klingman G I & J D Klingman Effects of immunosympathectomy on the superior cervival ganglion and other adrenergic tissues of the rat *Life Sci* 1965, 4 2171–2179

Maxwell, R A, A J Plummer F Schneider, H Povalski & A I Daniel Pharmacology of [2 (octahydro 1 azocinyl)-ethyl] guanidine sulfate (SU 5864) *J Pharmacol* 1960 128, 22–29

Pascoe J E The effects of acetylcholine and other drugs on the isolated superior cervival ganglion *J Physiol* (London) 1956 132 242–255

Quilliam, J P & D G Shand The selectivity of drugs blocking ganglionic transmission in the rat *Brit J Pharmacol* 1964, 23 273–284

Watson P J Drug receptor sites in the isolated superior cervical ganglion of the rat *Eur J Pharmacol* 1970 12, 183–193

Acta pharmacol et toxicol 1973, 32, 382–389

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The Effect of Guanethidine on the Noradrenaline Content of the Adult Rat Superior Cervical Ganglion

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Abstract The administration of guanethidine sulphate in doses above 10 mg/kg intraperitoneally for more than a week induced a considerable reduction (up to 80 %) of the noradrenaline content in the adult rat superior cervical ganglion With 5 mg/kg no changes were observed even after administration for 4 weeks Single injections of 5 and 20 mg/kg had no effect on the ganglionic noradrenaline. Following administration for 14 days in a dose of 20 mg/kg normalization of the ganglionic noradrenaline was complete within 8 days Following 40 mg/kg for 18 days recovery was only partial – probably due to irreversible degeneration of some ganglion cells The effect of guanethidine on the noradrenaline content in the superior cervical ganglion is different from its noradrenaline depleting effect in sympathetically innervated peripheral organs and is assumed to be secondary to chromatolytic changes in the ganglionic nerve cells induced by guanethidine some of these changes being irreversible following high doses for long periods of administration

Key words Guanethidine ganglia autonomic – noradrenaline

Guanethidine has been shown to deplete the stores of catecholamines in various organs – this depletion, however, is not considered as being the likely mechanism of action (survey by FURST 1967) Acute administration of guanethidine in the cat rabbit, and rhesus monkey partially depletes the catecholamines from the superior cervical ganglion (SPECTOR *et al* 1960, SANAN & VOGT 1962 SCHOEPKE *et al* 1965) A depletion was demonstrated histochemically in the rat following prolonged administration (JENSEN HOLM & JUUL 1970b, BURNSTOCK *et al* 1971a & b, ERANKO & ERANKO 1971a)

Pronounced morphological and ultrastructural changes in adult rat superior cervical ganglia following prolonged administration of guanethidine have been described previously (JENSEN-HOLM & JUUL 1970b & 1971) A nearly complete irreversible destruction of the sympathetic ganglion

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nerve cells by guanethidine has been demonstrated in newborn animals by different groups of investigators, originally by ERÄNKÖ & ERÄNKÖ (1971b), and in adult rats by BURNSTOCK *et al* (1971b)

The purpose of the present investigation has been to study quantitatively the effect of guanethidine at various dose levels and for varying lengths of time on the noradrenaline content of the adult rat superior cervical ganglion

Material and methods

Adult male Wistar rats weighing 200-300 g were used Guanethidine sulphate (ismelin®, CIBA) 10 mg/ml dissolved in saline was administered intraperitoneally once daily In the initial experiments the control rats received daily injections of saline 0.5 ml intraperitoneally, while in the remaining experiments they were not injected since no differences in the noradrenaline content of the superior cervical ganglion was observed between the groups The animals were killed by exsanguination under ether anaesthesia and the superior cervical ganglia quickly excised under the operating microscope and stored at 80° until use Pooled ganglia from two rats were homogenized in 1 ml of cold perchloric acid using a glass homogenizer

The noradrenaline (NA) content was determined fluorometrically following separation on a Dowex W-4X 200-400 mesh (Fluka) column sodium form (HÄGGENDAL 1963) and subsequent oxidation by iodine (LAVERTY & TAYLER 1968) The homogenate (adjusted to approximately pH 3 with potassium carbonate) was put on the Dowex column (internal diameter 2.7 mm length 65 mm (in water)) From the elution profile it was found that the NA was contained within 3 5 ml of the eluate the first 1.5 ml being discarded Apart from minor adjustments of volumes the oxidation procedure of LAVERTY & TAYLOR (1968) was strictly followed Fluorescence was measured by means of an Aminco-Bowman spectrophotofluorometer using microcuvettes reading emission at 480 nm, excitation at 400 nm (uncorrected instrument values) Excitation spectra were routinely recorded

The sensitivity of the method was 12 ng NA per analysis corresponding to 3 ng NA per ganglion The recovery of added NA varied from 75 to 100 % (mean value 84 %) Guanethidine did not interfere with the analysis Adrenaline and dopamine were not co-determined

Attempts to increase the sensitivity of the method by freeze drying the eluate from the Dowex column proved unsuccessful due to the destruction of NA the recovery of added ^{3}H NA being invariably higher than the recovery of NA measured fluorometrically

The statistical calculations are based on Student s t test

Results

A single intraperitoneal injection of guanethidine sulphate at 5 and 20 mg/kg did not significantly change the NA content of the superior cervical ganglia excised 1, 4, 8, 24, and 100 hours after administration The average mean NA content in 13 control rats was 26 ng/ganglion (S D 2 6), following 5 mg/kg between 22 and 27 ng/ganglion, and following 20 mg/kg between 22 and 26 ng/ganglion

The dose response curve for the NA lowering effect of guanethidine

14 days' administration is given in fig 1A The duration of the experiment was chosen according to other studies which indicated near maximal histological changes in the ganglia with only minor irreversible degeneration of the nerve cells (JUUL & SAND 1973) The time of discontinuation was chosen from fig 2B as the point of maximal NA depletion of guanethidine (4–8 hours) No effect was obtained by guanethidine 5 mg/kg, and a near maximal effect by 20–40 mg/kg.

Fig 1B represents the effect of guanethidine administration for 18 days followed by 63 days' discontinuation Only the NA values following 40 mg/kg are outside the normal range

The effect of increasing periods of administration appears from fig 2A The values were obtained following discontinuation for 24 hours to avoid the acute effects of guanethidine on NA as appears from fig 2B At a dosage of 5 mg/kg no changes were observed, even after 31 days With 20 mg/kg a significant decrease of approximately 45 % following administration for 14 days was obtained ($P < 0.01$) A near maximal decrease was obtained after administration for 1 month

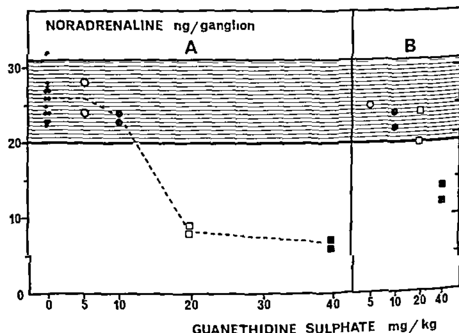


Fig 1 Noradrenaline content of rat superior cervical ganglia following intraperitoneal administration of guanethidine sulphate at various dose levels for 14 days followed by discontinuation for 4 hours (A) and for 18 days followed by discontinuation for 63 days (B) The normal range is hatched (mean value ± 2 S D) Each point represents the result of analysis of 4 pooled ganglia

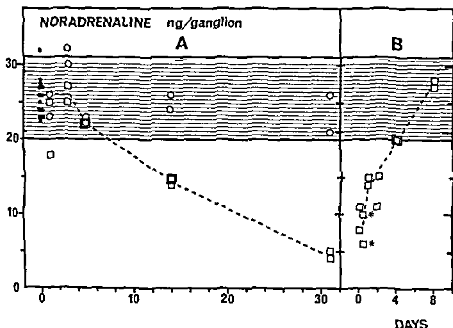


Fig 2 Noradrenaline content of rat superior cervical ganglia following intraperitoneal administration of guanethidine sulphate at 5 mg/kg (open circles) and 20 mg/kg (open squares) for varying lengths of time, discontinuation 24 hours (A) and discontinuation for varying lengths of time following administration for 14 days of 20 mg/kg (B) The 8 hours discontinuation values are indicated by asterisks The normal range is hatched (mean value ± 2 S D) Each point represents the result of analysis of 4 pooled ganglia

Fig 2B represents a discontinuation experiment following administration for 14 days at a dose of 20 mg/kg Normalization of the NA values occurred within 8 days

Fig 3 represents an estimation of the extent of irreversible degeneration of the ganglion nerve cells following administration of guanethidine at different dose levels and for varying length of time Part of the figures forming the basis of this figure are taken from JUUL & SAND (1973)

Discussion

The present investigation demonstrates that high doses of guanethidine for several days lower the NA content of the adult rat superior cervical ganglion whereas a single injection is without effect A single dose of guanethidine sulphate of 15–20 mg/kg lowered the NA content up to 66 % in the superior cervical ganglion of the rabbit and cat (SPECTOR *et al* 1960, SANAN & VOGT 1962) and of the rhesus monkey (SCHOEPKE *et al* 1965) The discrepancy

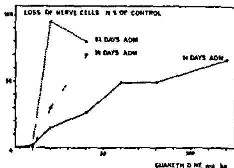


Fig 3 Estimated loss of nerve cells of rat superior cervical ganglia following treatment with guanethidine sulphate at various dose levels for 14, 30 and 63 days The figures are based on cell counts in histological specimens and corrected for differences in ganglionic size Part of the figures are taken from JUUL & SAND (1973)

between these findings and the present results may be due to differences between the species

The NA lowering effect of guanethidine in the superior cervical ganglion differs from the NA depleting ("reserpine like') effect in sympathetically innervated peripheral organs (survey by FURST 1967) This effect can be produced by a single injection of a low dose (1–5 mg/kg (ARNOLD *et al* 1963)), the effect occurs rapidly (within hours) and prolonged administration of 5–10 mg/kg results in a nearly complete disappearance of NA (CASS & CALLINGHAM 1964, BURNSTOCK *et al* 1971a) In all these aspects the effect of guanethidine on the rat superior cervical ganglion differs (fig 1 and 2), and this may at least partially be due to different compartments of NA in the peripheral axons and the sympathetic ganglia It has been demonstrated that NA is located in the storage granules to a major extent in the peripheral axons than in the sympathetic ganglia (FISCHER & SNYDER 1965, CHUBB *et al* 1972) Differences in the sensitivity between the peripheral organs and sympathetic ganglia have also been described concerning the NA depleting activity of reserpine (NORBERG 1965)

It has been postulated that the NA depleting effect of guanethidine could be explained on the basis of a replacement by guanethidine at a molecular level (CHANG *et al* 1965) Although a correlation between the guanethidine content in the superior cervical ganglion (JUUL & SAND 1973) and the depletion is also found in the present investigation it cannot be taken as evidence for this hypothesis since the compartmental distribution of both guanethidine and NA in the ganglia is not definitely known and may differ between the peripheral axons and the ganglia (FISCHER & SNYDER 1965, CHUBB *et al*

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changes in the superior cervical ganglion in the conditions used in the present investigation (JENSEN HOLM & JUUL 1970a, b & 1971, ERÄNKÖ & ERÄNKÖ 1971a) In the light microscope chromatolysis of the nerve cells was observed, and by the electron microscope there was a pronounced dilatation of the mitochondria in the nerve cells A pronounced loss of cholinesterases, preferably from the nerve cell cytoplasm, and a depletion of histochemically demonstrated catecholamines was also found

BURNSTOCK *et al* (1971a) using a histochemical method found a reduced catecholamine content in the adult rat superior cervical ganglion after administration for 4–18 weeks of 5 mg/kg In the present investigation a dose of 5 mg/kg did not produce any changes aftes 4 weeks' administration but it is possible that a prolongation of the administration could have resulted in a lowering of the NA content (fig 2A)

Since the doses and the duration of administration necessary to reduce the NA content are similar to those required to induce morphological changes we find it reasonable to assume that the loss of NA is secondary to the chromatolysis Similar changes in the NA content following the chromatolysis induced by postganglionic axotomy have been reported by other investigators e g ERÄNKÖ & HARKONEN (1965) The time course of these changes, however, is different since normal NA levels are not reached until 3 months after axotomy

ERÄNKÖ *et al* (1972) considered it unlikely that guanethidine could have a direct cytotoxic effect on the ganglion nerve cells since nerve cells in cultures of sympathetic ganglia of newborn rats are apparently not influenced by the addition to the medium of high concentrations of guanethidine (up to 0 12 mM) It may be questioned however, whether identical properties of nerve cells in cultures of ganglia and of similar cells functioning *in situ* can be assumed Thus the nerve cells in control cultures did not contain appreciable amounts of histochemically demonstrable catecholamines

BURNSTOCK *et al* (1971b) described an almost complete irreversible degeneration of the ganglion cells accompanied by an almost complete disappearance of the NA fluorescence in adult rats after the intraperitoneal administration of guanethidine sulphate at 25 30 mg/kg for 6 weeks A similar chemical sympathectomy is much more easily induced in newborn animals (ERÄNKÖ & ERÄNKÖ 1971b) In the present investigation the loss of ganglionic NA cannot solely be explained as the result of irreversible degeneration of the nerve cells Fig 2 and 3 demonstrate that a loss of NA of 40–60 % (following guanethidine sulphate 20 mg/kg for 14 days) is accomplished at an estimated loss of ganglion nerve cells of only 15 % Similarly the almost complete reversibility of the NA changes following guanethidine 20 mg/kg for 14 days as seen in fig 1B and 2B and the partial reversibility of the changes following 40 mg/kg for 14 days (fig 1B) contradict this pos-

sibility However, fig 3 also demonstrates that higher doses and/or longer periods of administration result in a much more pronounced sympathectomy in agreement with the findings of BURNSTOCK *et al* (1971b) The incomplete recovery of the NA in fig 1B following 40 mg/kg for 18 days (52 % of the control value) may thus be caused by irreversible degeneration of some of the ganglion cells, the loss of nerve cells following 40 mg/kg for 2–4 weeks being estimated to be 30–70 % (fig. 3)

The actual mechanism underlying the NA depleting activity of guanethidine in sympathetic ganglia is unknown and cannot be deduced from the present experiments

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REFERENCES

Arnold A J P McAuliff S D Sobell & S Archer Dose response effects on norepinephrine depletion of reserpine and of guanethidine in bretylium pretreated rats *Biochem Pharmacol* 1963 **12** 1231–1232

Burnstock G A E Doyle B J Gannon J F Gerkens T Iwayama & M L Mashford Prolonged hypotension and ultrastructural changes in sympathetic neurones following guanacline treatment *Eur J Pharmacol* 1971a **13** 175 187

Burnstock G B Evans B J Gannon J W Heath & V James A new method of destroying adrenergic nerves in adult animals using guanethid ne *Br t J Pharmacol* 1971b **43** 295–301

Cass R & B A Callingham Some effects of drugs which influence sympathetic transmission on t ssue catecholamines in the rat *B ochem Pharmacol* 1964 **13** 1619–1625

Chang C C E Costa & B B Brodie Interaction of guanethidine with adrenerg c neurons *J Pharmacol* 1965 **147** 303–312

Chubb I W W P De Potter & A F De Schaepdryver The storage of noradrenaline in sympathetic ganglia *Life Sci* 1972 **11** 323–333

Eranko L & O Eranko Effect of guanethidine on nerve cells and small intensely fluorescent cells in sympahetic ganglia of newborn and adult rats *Acta pharmacol et toxicol* 1971a **30** 403–416

Eranko O & L Eranko Histochemical evidence of chemical sympathectomy by guanethidine in newborn rats *Histochem J* 1971b **3** 451–456

Eranko O & M Harkonen Effect of axon division on the distribution of noradrenal ne and acetylcholinesterase in sympathetic neurons of the rat *Acta phys ol scand* 1965, **63** 411–412

Eranko L C Hill O Eranko & G Burnstock Lack of toxic effect of guanethid ne on nerve cells and small intensely fluorescent cells in cultures of sympathet c ganglia of newborn rats *Bra n Res* 1972 **43** 501 513

Fischer J E & S Snyder Disposition of norepinephr ne H³ in sympathetic ganglia *J Pharmacol* 1965 **150** 190–195

Furst C I The biochemistry of guanethidine *Adv Drug Res* 1967 **4** 133–161

Haggendal, J An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissues *Acta physiol scand* 1963, **59**, 242–254

Jensen Holm, J & P Juul The effects of guanethidine, pre- and postganglionic nerve division on the rat superior cervical ganglion cholinesterases and protein *Acta pharmacol et toxicol* 1970a, **28**, 270–282

Jensen Holm, J & P. Juul The effects of guanethidine, pre- and postganglionic nerve division on the rat superior cervical ganglion cholinesterases and catecholamines (histochemistry), and histology *Acta pharmacol et toxicol* 1970b, **28**, 283–298

Jensen Holm, J & P Juul Ultrastructural changes in the rat superior cervical ganglion following prolonged guanethidine administration *Acta pharmacol et toxicol* 1971, **30**, 308–320

Juul, P & O Sand Guanethidine determination in sympathetic ganglia *Acta pharmacol et toxicol* 1973, **33**, in press

Laverty, R & K M Taylor The fluorometric assay of catecholamines and related compunds Improvements and extensions to the hydroxyindole technique *Anal Biochem* 1968, **22**, 269–279

Maître, L & M Staehelin Guanethidine uptake and noradrenaline depletion in noradrenaline storage particles of the rat heart *Biochem Pharmacol* 1971, **20**, 1233–1242

Norberg, K A Drug induced changes in monoamine levels in the sympathetic adrenergic ganglion cells and terminals *Acta physiol scand* 1965, **65**, 221–234

Sanan, S & M Vogt Effect of drugs on the noradrenaline content of brain and peripheral tissues and its significance. *Brit J Pharmacol* 1962, **18**, 109–127

Schoepke, H G, H D Brondyk, L H Wiemeler & J L Schmidt Cardiovascular pharmacology of tertiary octyl guanidine (BP 1184) *Arch int Pharmacodyn* 1965, **153**, 185–204

Spector, S, R Kuntzman, C Hirsch & B B Brodie Norepinephrine content of various ganglia after reserpine guanethidine and JB 516 *Pharmacologist* 1960, **2**, 94

Acta pharmacol et toxicol 1973, 32, 390–400

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The Decapitated Rat as a Test Object for the study of Hypo- and Hyperthermic Acting Compounds*

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Abstract The effects of hyper and hypothermic acting compounds on the rectal and skin temperature, oxygen consumption, and heart rate were studied in the *decapitated rat Noradrenaline infusion* (2 µg/kg/min) *caused thermogenesis*, increased oxygen consumption, tachycardia, and vasoconstriction 2,4-Dinitrophenol (2 × 2 5 mg/kg intraperitoneally) and sodium salicylate (150 mg/kg intraperitoneally), which cause an uncoupling of the oxydative phosphorylation, caused thermogenesis and increased oxygen consumption but no significant changes in the skin temperature or heart rate The effects of noradrenaline but not those of 2 4 dinitrophenol and sodium salicylate could be blocked or reduced by the administration of propranolol and phentolamine Thus the thermogenesis caused by noradrenaline could be differentiated from that caused by uncouplers of oxydative phosphorylation The metabolic poison, iodoacetic acid reduced or prevented the noradrenaline induced thermogenesis when given as a pretreatment (30 mg/kg intraperitoneally) 60–75 min before the noradrenaline infusion Chlorpromazine (10 mg/kg intraperitoneally) did not influence the noradrenaline induced thermogenesis either when given as a pretreatment or when given during the infusion It is concluded that the decapitated rat is useful for the study of the mechanism of action of hyper and hypothermic acting compounds

Key words Body temperature – decerebrate state – rats – norepinephrine – dinitrophenol – sodium salicylate – sympatholytics – iodoacetates – chlorpromazine

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When new test compounds or well-known drugs are administered to experimental animals the body temperature is often seen to decrease or increase The complexity of the physiological thermoregulation (CREMER & BLIGH 1969) makes the analysis of the mode of action of a hypo- or hyperthermic acting compound rather difficult The main question to be answered is whether the effect is mediated via the thermo-regulatory centre

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*) Some of these results were presented at the 1 Congress of the Hungarian Pharmacological Society, Budapest, October 18th–22nd, 1971

in the hypothalamus, or it is brought about by a direct peripheral action The decapitated rat in which the hypothalamic regulation is eliminated represents a purely peripheral test object and may therefore be useful for the study of the site of action of hypo or hyperthermic compounds This paper describes the decapitated rat as a new set up, and the results obtained with some reference substances are given

Method

Male Wistar rats weighing 200–350 g were used Anaesthesia was induced with ether or pentobarbital sodium (mebumal natrium NFN nembutal®) 75 mg/kg intraperitoneally The decapitation was carried out according to the principle described by KREPPEL (1959) for cats The vagus nerves were cut through a midline incision ventrally in the neck A polyethylene tube (Clay Adams PE 50) was implanted into one or both of the jugular veins The trachea was cannulated with a branched glass cannula and both carotid arteries ligated A polyethylene tube for intraperitoneal injections was inserted into the abdomen The neck was clamped with a powerful hemostat (Rochester Pean) just above the tracheotomy and the head cut off The tracheal cannula was connected to a miniature respiratory pump (Palmer) The pump was connected to a bell spirometer filled with oxygen and the expired air was passed through calcium chloride and soda asbestos (Merck) before reentering the spirometer Following the operation the animals were allowed to stabilize for 1 2 hours When ether was used for anaesthesia the preparation was respirated in an open system during this period in order to eliminate the ether The animal was placed in a thermo insulating box made of polystyrol and thermocouple applicators were placed in the rectum in the skin of the tail and on the surface of the left forepaw A fourth thermocouple applicator measured the temperature in the box The temperatures were registered by means of a temperature recorder (Type Z 8 Ellab Elektrolaboratoriet Copenhagen) In order to control that the preparation was alive and to study the effects of the test substances on the heart rate ECG electrodes were placed subcutaneously and connected to a Mingograf 12 electrocardiograph (Elema Stockholm)

The term hypothermic is used for substanses reducing the heat production even to a minor degree and the term "hyperthermic for substances reducing or reversing the spontaneous decrease in the rectal temperature of the preparation

Test substances 2 4-Dinitrophenol sodium salicylate (natrii salicylas Ph Nord) noradrenaline bitartrate (noradrenalini bitartras Ph Nord) propranolol hydrochloride (propranololi chloridum NFN inderal ®) phentolamine methanesulphonate (fentolamini methansulfonas NFN regitin®) iodoacetic acid and chlorpromazine hydrochloride (chlorpromazini chloridum NFN, klorpromazin Novo) The doses given in the text refer to these compounds when not otherwise stated Solutions were prepared with sterile pyrogen free saline 2 4 Dinitrophenol was dissolved by the addition of sodium bicarbonate The volume injected per dose was 2 ml/kg Noradrenaline was infused at a rate of 2 μg/kg/min calculated as the base The volume infused was 0 04 ml/min

Results

All substances were tested with both ether and pentobarbital anaesthesia No differences in the responses were observed However, the start-

Table 1.

Effects on rectal temperature of saline, 2,4-dinitrophenol, sodium salicylate and noradrenaline with and without pretreatment with iodoacetic acid The figures are mean values ± S D The figures in the brackets indicate the lowest and higest values observed.

	Saline 2 ml/kg intraperitoneally n = 14	2,4-Dinitrophenol 2 × 2.5 mg/kg intraperitoneally n = 12	Sodium salicylate 150 mg/kg intraperitoneally n = 8	Noradrenaline 4 µg/kg/min intravenously n = 41	Noradrenaline after iodoacetic acid 30 mg/kg intraperitoneally n = 10
A Δt_{rect}/10 min before treatment	−0 24 ± 0 14	−0 27 ± 0 14	−0 16 ± 0 11	−0.23 ± 0 09	−0 21 ± 0 08
	(−0 50 − 0 00)	(−0 50 − −0 05)	(−0 35 − −0 06)	(−0 50 − −0 05)	(−0 30 − −0 05)
B Δt_{rect}/10 min after treatment	−0 24 ± 0 14	0 04 ± 0 15	0 05 ± 0 11	0.21 ± 0 13	0 04 ± 0 10
	(−0 50 − 0 00)	(−0 30 − 0 25)	(−0 15 − 0 20)	(0 00 − 0 50)	(−0 15 − 0 24)
B–A	0 00 ± 0 03	0 31 ± 0 14	0 21 ± 0 08	0 44*) ± 0 14	0 25*) ± 0 08
	(−0 05 − 0 05)	(0 10 − 0 53) P < 0 005	(0 10 − 0 33) P < 0 005	(0 20 − 0 80) P < 0 001	(0 15 − 0 43) P < 0 001

*) The difference between these values is statistically significant, P < 0 001

Table 2

Effects on rectal temperature of phentolamine, propranolol and chlorpromazine given during an infusion of noradrenaline The figures are mean values ± S D The figures in the brackets indicate the lowest and highest values observed

	Phentolamine 2 5 mg/kg intravenously n = 8	Propranolol 1 mg/kg intravenously n = 6	Chlorpromazine 10 mg/kg intraperitoneally n = 11
A			
Δt_{rect}/10 min before	0 28 ± 0 11	0 23 ± 0 13	0 17 ± 0 10
treatment	(0 10 – 0 40)	(0 10 – 0 40)	(0 05 – 0 40)
B			
Δt_{rect}/10 min after	0 04 ± 0 12	–0 04 ± 0 09	0 16 ± 0 11
treatment	(–0 10 – 0 20)	(–0 15 – 0 07)	(–0 05 – 0 40)
	–0 24 ± 0 04	*–0 28 ± 0 13*	*–0 02 ± 0 11*
B – A	(–0 31 – –0 20)	(–0 48 – –0 15)	(–0 30 – 0 10)
	P < 0 001	P < 0 005	N S

ing level of the skin temperature was generally somewhat higher when ether was used

A survey of the results concerning the rectal temperature is given in tables 1 and 2 The slope of the curves is expressed as the temperature change per 10 min, an interval in which the curves can be considered to be linear The value B–A represents the effect of the substance given

A Control experiments (n=14) When the decapitated rat is left untreated the rectal and skin temperatures as well as the oxygen consumption always decrease spontaneously Fig 1 shows that the administration of saline does not alter this decrease either when performed intraperitoneally or intravenously as an infusion The heart rate is practically constant throughout the experiment

B Experiments with hyperthermic acting compounds (table 1)

2 4 Dinitrophenol (n=12) In the intact rat 2,4 dinitrophenol increases the body temperature significantly when given in doses of 20–30 mg/kg intraperitoneally (own observation) This is also the case in the decapitated rat The results of such an experiment are given in fig 2 The decrease in

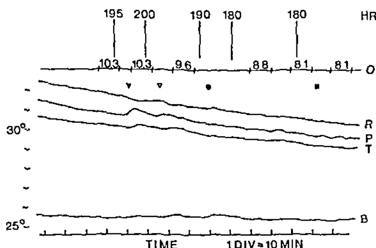


Fig 1 Control experiment ▼ = saline 2 ml/kg intravenously ▽ = saline 2 ml/kg intraperitoneally ● = saline infusion 0 04 ml/min intravenously ■ = end of infusion HR = heart rate in beats/min O = oxygen consumption in ml/kg/min R = rectal temperature P = skin temperature of the left forepaw T = skin temperature of the tail B = temperature in the thermo insulating box

rectal temperature stops when a total dose of 5 mg/kg administered intraperitoneally is given The skin temperature changes in parallel with the rectal temperature The oxygen uptake goes up whereas the heart rate is largely unaffected during the course of the experiment

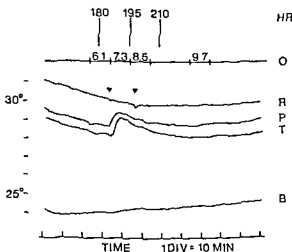


Fig 2 Effects of 2,4-dinitrophenol ▼ = 2,4-dinitrophenol 2 5 mg/kg intraperitoneally Letter code see fig 1

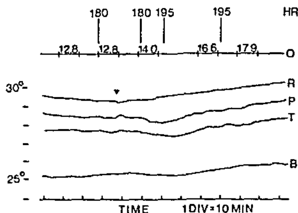


Fig 3 Effects of sod um sal cylate (▼) 150 mg/kg intraperitoneally Letter code see fig 1

Sodium salicylate (n=8) This substance is antipyretic in normal rats made febrile with yeast injections A complete abolition of the hyperpyretic response to yeast is obtained with 75 mg/kg intraperitoneally In normo thermic intact rats doses above 500 mg/kg cause an increase in rectal temperature (own observation) Fig 3 shows the effects of sodium sali cylate in the decapitated rat A dose of 150 mg/kg intraperitoneally results in an increase in rectal as well as in skin temperatures and oxygen con sumption The heart rate is almost unchanged by this dose

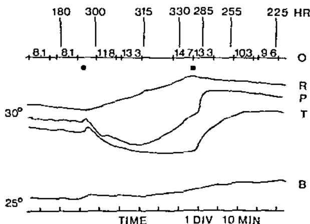


Fig 4 Effects of an ntravenous infus on of noradrenal ne (●) 2 µg/kg min calculated as the base Infus on rate 0 04 ml/min ■ = end of infusion Letter code see f g 1

Noradrenaline (n=41) Infusion of noradrenaline increases the body temperature as well as the heart rate and the oxygen uptake (fig 4) The temperature of the skin which is a result of a peripheral vasocontriction and an augmented blood flow due to the elevated blood pressure may either decrease or increase An increased skin temperature is normally seen at infusion rates below 2 µg/kg/min, whereas infusion rates higher than this value most often result in a lowered skin temperature At the end of the infusion the effects of the noradrenaline disappear immediately

In order to characterize the response to noradrenaline the effects of the α-receptor blocking agent phentolamine and the β-receptor blocking agent propranolol on the noradrenaline response were studied (table 2) Phentolamine (2 5 mg/kg intravenously) (n=8) reduces the noradrenaline induced heat production and the increase in oxygen consumption (fig 5) The skin temperature goes up indicating a peripheral vasodilatation Phentolamine was incapable of normalizing the increased heart rate produced by the noradrenaline infusion

The β adrenergic blocking agent, propranolol (1 mg/kg intravenously) (n=6) reduces the noradrenaline induced increase in heat production and oxygen consumption (fig 6) In contrast to phentolamine, propranolol partly normalized the noradrenaline induced increase in heart rate The skin temperature, however, continued to decrease as long as noradrenaline was infused The peak seen in the paw temperature at the time of the injection is believed to be an unspecific response to the injection (compare fig 1)

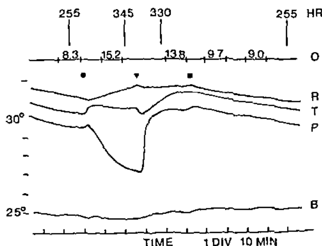


Fig 5 Effects of an intravenous injection of phentolamine (▼) 2 5 mg/kg given during an infusion of noradrenaline (●) ■ = end of infusion Infusion data see fig 4 Letter code see fig 1

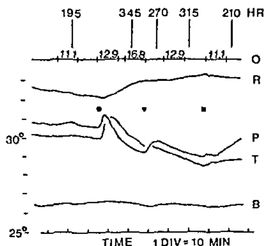


Fig 6 Effects of an intravenous injection of propranolol (▼) 1 mg/kg given during an infusion of noradrenaline (●) ■ = end of infusion Infusion data see fig 4 Letter code see fig 1

C Experiments with hypothermic acting compounds (tables 1 and 2)

Iodoacetic acid (n=10) The metabolic poison, iodoacetic acid, causes hypothermia in intact rats when given in doses above 40 mg/kg intraperitoneally (own observation) In the decapitated rat this substance does not accelerate the spontaneous decline in rectal temperature, nor does it influence the noradrenaline induced thermogenesis when injected during the

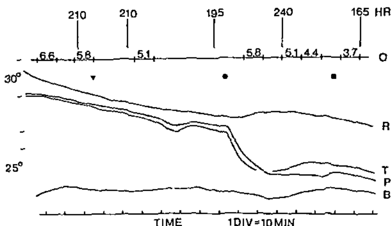


Fig 7 Effects of iodoacetic acid (▼) 30 mg/kg given intraperitoneally 70 min before the infusion of noradrenaline (●) ■ = end of infusion Infusion data see fig 4 Letter code see fig 1

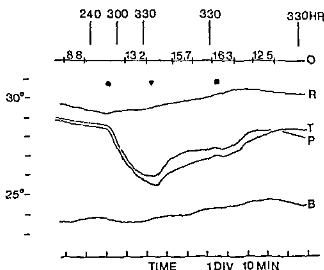


Fig 8 Effects of an intraperitoneal injection of chlorpromazine (▼) 10 mg/kg given during an infusion of noradrenaline (●) ■ = end of infusion Infusion data see fig 4 Letter code see fig 1

infusion When, however, the preparation is pretreated with iodoacetic acid 30 mg/kg intraperitoneally 60–75 min before the infusion the thermogenic response to noradrenaline is markedly reduced as is the increase in oxygen consumption The tachycardiac response to noradrenaline is reduced whereas the peripheral vasoconstriction appears not to be influenced by the pretreatment (fig 7)

Chlorpromazine (n=11) This neuroleptic drug depresses the body temperature in intact animals In rats the threshold dose is 2 5 mg/kg intraperitoneally, and a dose dependent aggravation of the hypothermia is seen with 5 and 10 mg/kg (own observation) In the decapitated rat the spontaneous fall in body temperature is uninfluenced by chlorpromazine Nor did chlorpromazine significantly depress the heat production induced by noradrenaline even when given in doses up to 10 mg/kg intraperitoneally The tachycardia is uninfluenced and the oxygen consumption increases during the whole infusion as is seen when noradrenaline is infused alone (compare fig 4) On the contrary the vasoconstrictory response to noradrenaline in the skin is reduced (fig 8)

Discussion

Two different mechanisms of hyperthermic action could be demonstrated in this study 1) The catecholamine response i e thermogenesis accom-

panied by tachycardia, vasoconstriction, and increased oxygen uptake which could be reduced or blocked by the administration of α- and β-adrenergic blocking agents 2) The response to 2,4-dinitrophenol and sodium salicylate, two uncouplers of oxydative phosphorylation (BRODY 1956), consisting of thermogenesis, increased oxygen uptake, slightly elevated heart rate, and a slight increase in skin temperature probably caused by the general warming of the preparation This response could not be blocked by phentolamine or propranolol

Concerning the effect of phentolamine on the noradrenaline induced thermogenesis it could be argued that the reduced rectal temperature after phentolamine may be caused by an increased heat dissipation due to the dilatation of the skin vessels This is, however, not the case as we have observed that the vasodilatation may appear 5 min later than the effect on the rectal temperature

Two substances, iodoacetic acid and chlorpromazine, which are hypothermic in intact rats were studied Iodoacetic acid was also active against noradrenaline induced changes in the decapitated rat when given as a pretreatment 60–75 min before the noradrenaline infusion

As the noradrenaline induced vasoconstriction is uninfluenced by the given dose of iodoacetic acid (30 mg/kg intraperitoneally) the reduction in thermogenesis is not likely to be due to an α receptor blockade The results given in fig 7 are more in the line with those obtained with propranolol On the other hand, when larger doses of iodoacetic acid are given or the pretreatment period is prolonged, all responses to noradrenaline are blocked As iodoacetic acid blocks glycolysis this property is more likely to be the causative factor in the antihyperthermic response

In the case of chlorpromazine no significant reduction in noradrenaline induced thermogenesis was seen, either when chlorpromazine was given as a pretreatment or when given during the noradrenaline infusion In some experiments no change in the skin temperature occurred, and in others, in which the temperature of the skin rose as a response to noradrenaline, it was lowered by chlorpromazine An explanation for this may be that chlorpromazine reduces the perfusion rate in the skin due to its blood pressure reducing effect Thus chlorpromazine despite being an α-receptor blocking agent (GOKHALE *et al* 1964, WEBSTER 1965) does not seem to exert its hypothermic effects in normal animals by blocking the peripheral α receptors Further studies on this problem are in progress In fig 8 it can be seen that the heat production and the tachycardia persist for some time after the infusion has been stopped This observation is in accordance with the results of studies with the spinal vagotomized cat reported by MARTIN *et al* (1960), who found prolonged pressor responses to noradrenaline after chlorpromazine

The results reported above indicate that it is possible to induce thermogenesis in the decapitated rat, an animal preparation in which the influence of the central thermoregulatory centre can be disregarded This means that substances acting on the core temperature in this preparation must have a purely peripheral mechanism of action It should, however, be kept in mind that a positive response with this technique does not exclude the presence of an additional central site of action in the intact rat

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REFERENCES

Brody, T M Action of sodium salicylate and related compounds on tissue metabolism *in vitro* *J Pharmacol exp Therap* 1956 **117** 39–51

Cremer J E & J Bligh Body temperature and responses to drugs *Brit Med Bull* 1969 **25** 299–306

Gokhale S D O D Gulati & H M Parikh An investigation of the adrenergic blocking action of chlorpromazine *Brit J Pharmacol* 1964 **23** 508–520

Kreppel E Ein Dekapitationsinstrument für Katzen *Med exp* 1959 **1** 361 363

Martin W R J L Riehl & K R Unna Chlorpromazine III The effects of chlorpromazine and chlorpromazine sulfoxide on vascular responses to L epinephrine and levarterenol *J Pharmacol exp Therap* 1960 **130** 37–45

Webster R A The antiadrenaline activity of some phenothiazine derivatives *Brit J Pharmacol* 1965 **25** 566–576

Acta pharmacol et toxicol 1973, 32, 401–407.

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Distribution and Excretion of Enibomalum (NFN) in Rats

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Abstract Rats were given enibomal (60 mg/kg) intravenously in a single dose Groups of animals were then killed after 15, 30, 60, 120, 180 and 240 min respectively, and the concentrations of enibomal determined In the plasma, liver and muscles the concentration was reduced between 15 and 30 minutes after the injection, after which it remained at a fairly constant level until one hour after the injection The concentration then fell again in the plasma at a rate corresponding to a half value period of about 2 hours In the brain a steep fall was seen during the first hour, after which the concentration fell at a slow rate 15 minutes after the injection the concentration was nearly the same in the plasma and fatty tissue Thereafter it rose steeply in the fat, to four times the value in the plasma within the first hour Finally the fat concentration fell, to reach a level of about 40 per cent of the maximum value four hours after the injection In urine of rats collected over 72 hours the following 5 isopropyl barbituric acid derivates (B) were detected 1 methyl 5 acetonyl B, 5 acetonyl B, and 1 methyl 5 (2 oxo-3 hydroxypropyl) B About 20 per cent of the administered dose was recoverable in the urine The kinds and amounts of metabolites were the same as previously found in human urine Neither enimobal nor the metabolites were detectable in the bile or in the small intestinal contents within the first four hours after the injection

Key words Enibomalum – barbiturates – distribution – excretion

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The fate of the thiobarbiturates in the organism and in particular their deposition, has been the subject of numerous investigations The fate of the N-substituted barbituric acid compounds, on the other hand, has been studied by only a few investigators

In a previously published paper dealing with the excretion of enibomal in man (Ravn Jonsen 1970) it was seen that metabolites were detectable for several days after the administration of the drug Owing to the low lipid-solubility and consequent high clearance of the breakdown products it was assumed that the unchanged enibomal was disposited For the

finding out whether this hypothesis is correct, an investigation was made on the distribution of emborнalum in rats at different intervals following intravenous injection To clarify whether the metabolic pattern of rats resembles that of man, urine samples from rats were analyzed to find out which kinds of metabolites were present To determine whether embomal is excreted into the intestinal tract the bile and the small-intestinal contents were analyzed for both embomal and its metabolites

Material and Methods

Distribution Six groups consisting of eight female rats each (165–220 g) were given intravenous injections of embomal sodium (narcodorm ®) in amounts corresponding to 60 mg embomal sodium per kilogram The animals were killed after 15, 30, 60 120 180 and 240 minutes respectively The thoracic cavity was opened, and about 4 ml of heart blood was transferred to a heparinized glass tube

The liver, brain muscles of the hindlegs, and fatty tissue from the abdominal cavity were excised Finally, the small intestine was taken out and divided into two parts an upper part in which the contents were still of a liquid consistency, and a lower part in which the contents were more solid The two parts were emptied seperately by thorough washing with water, and examined for the presence of embomal and its metabolites

Excretion, a Urine Eight rats received injections as described under distribution The animals were placed in metabolism cages and their urine was collected over 72 hours During the first 30 hours samples from 6 hour periods were collected, and then samples from 14-hour periods The cages used were thereafter washed twice with water, and the washings added to the collected urine Finally, the cages were washed twice with metanol This was evaporated and the collected urine together with the wash water were added to the residues of evaporation

b Bile Four rats (190 g) received injections as described under distribution The bile duct was surgically exposed and cannulated with polyethylene tubing (inner diameter 0 75 mm) The collection of the bile was started approximately 15 min after the injection and continued for four hours The samples were taken at hourly intervals The rats were kept anaesthetized with halothane (NFN) and the loss of water caused by the collection of the bile was compensated for by intraperitoneal injections of 0 9 % sodium chloride solution The flow of the bile was 460–870 µl per hour

Analytical methods

Liver The whole liver is homogenized and the largest possible amount weighed (7–10 g) After addition of tartaric acid to bring about an acid reaction the mixture is extracted three times with a large volume of chloroform in a separating funnel The dried and filtered chloroform extracts are evaporated to dryness and extracted three times with boiling water The pH of the filtered and cooled aqueous phase is adjusted to 7 5 This is followed by extraction three times with a large volume of chloroform The aggregate chloroform extracts are dried, filtered and evaporated to dryness

Spectrophotometry The residue is dissolved in 10 ml of chloroform and extracted with 2 50 ml of phosphate buffer at pH 10 0 (phase A) The chloroform phase is transferred to another separating funnel washing being performed with 2 ml of chloro-

form This is followed by extraction with 3 00 ml of phosphate buffer at pH 12.0 (phase B) Phase B is analyzed within the range of 200–350 nm Measurement first at pH 12 gives an absorption curve suggesting the presence of enibomal This is followed by measurement at acid reaction and at pH 14 If the test solution becomes cloudy at acid reaction the solution is then filtered through a glass filter (G4) The barbituric acid concentration is then calculated on the basis of the measurement at pH 14 and at acid reaction

Recovery after addition of 200 μg enibomal to 10 g liver (20 μg/g) 78 per cent

Muscle The excised muscle specimen (2 5–3 0 g) is homogenized after addition of tartaric acid and the required volume of chloroform This is extracted three times with a large volume of chloroform in a separating funnel and continued as indicated for the liver

Recovery after addition of 60 μg enibomal to 3 g muscle (20 μg/g) 87 per cent

Blood The heparinized blood is centrifuged (3000 cpm) and 1 50–2.00 ml of plasma carefully withdrawn The plasma is thereafter extracted three times with a large volume of chloroform at the plasma s own pH Then continued as indicated for the liver

Recovery after the addition of 40 μg enibomal to 2 ml of plasma (20 μg/ml) 84 per cent

Fatty tissue The specimen (1 0–3 0 g) is weighed and 5 00 ml of phosphate buffer at pH 12 0 is added Homogenisation follows in ice water bath Another 2×5 00 ml of buffer are added during the homogenisation The homogenate is centrifuged and left in ice water bath for 5 10 minutes This causes division into three phases an upper fairly solid lipid phase a middle milky aqueous phase and a lower phase of heavy, solid constituents 10 00–12 00 ml of the middle phase are withdrawn and this aliquot is rendered acid by adding 8 N hydrochloric acid This is extracted three times with a large volume of ether The pooled dried and filtered ether phases are evaporated to dryness The residue is dissolved in 10 ml of chloroform and used for spectrophoto metric analysis

Recovery after the addition of 120 μg to 2 g fat (60 μg/g) 71 per cent

Brain The whole brain (1 5 g) is weighed and homogenized after the addition of 10 ml of water After the addition of 8 N hydrochloric acid to bring about an acid reaction the material is extracted three times with a large volume of ether

The pooled dried and filtered ether extracts are evaporated to dryness The residue is extracted first with 10 ml of 0 1 N sulphuric acid and then twice with 10 ml of water The pH of the pooled aqueous extract is adjusted to 7 5 This is followed by extraction three times with large volumes of ether After evaporation of the combined ether phases the residue is dissolved in 10 ml of chloroform and used for spectrophotometric analysis Recovery after the addition of 30 μg to 1 5 g brain (20 μg/g) 60 %

Urine The mixture consisting of the residue from the methanol washing urine, and wash water is treated for two hours with β glucoronidase at 37°

Then chemical analysis is performed as described previously (Ravn Jonsen 1970)

Small intestinal contents The wash fluids from the upper and lower sections respec tively of the small intestine are treated with β glucuronidase as described for urine The fluid is then extracted three times with large volumes of ether at acid reaction (pH about 2) The ether is removed by evaporation and 2×1/20 residue is used for two-dimensional thin layer chromatography (TLC) To one aliquot any possible enibo mal metabolites (Ravn Jonsen 1970) are added as reference standards The chromatograms are developed by systems A and C (Ravn Jonsen 1970) Following the action of ammonia vapour the chromatogram is studied in UV light of wavelength 254 nm and developed with a mercuric sulphate diphenyl carbazone reagent

Bile The bile is treated with β glucuronidase and extracted as described for the small intestinal contents

1/20 is applied to TCL and the chromatogram is developed and sprayed as described *for the small intestinal contents*

Results

Three metabolites were detectable in the urine after intravenous administration of emibomal to rats The excretion of these was concluded within about 48 hours (fig 1) A total of about 20 per cent of the injected emibomal was recoverable The amount recovered was excreted as 1-methyl 5 (2-oxo 3′-hydroxypropyl) 5-isopropylbarbituric acid (VI), 1-methyl 5 acetonyl 5-isopropylbarbituric acid (II), and 5 acetonyl-5 isopropylbarbituric acid (VIII) The compounds found were present in the following amounts VI about 11 %, II about 1 %, and VIII about 8 % Non-converted emibomal was not detected

VI and II were demonstrable in the urine within six hours of the injection while VIII was not seen until after this interval

TLC analysis of both the bile and the small-intestinal contents within four hours of the injection revealed no signs of the presence of emibomal or of any possible metabolites (RAVN-JONSEN 1970)

The detection limit on the chromatogram was 5–10 μg The excretion of

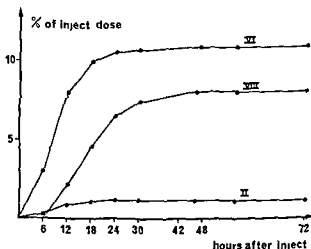


Fig 1 The amounts of metabolites excreted in the urine of rats after a single dose of emibomal sodium (NFN) (60 mg/kg) injected intravenously VI 1 methyl 5 (2 oxo 3 hydroxy) 5 isopropylbarbituric acid VIII 5 acetonyl 5 isopropylbarbituric acid *II* 1 methyl 5 acetonyl 5 isopropylbarbituric acid

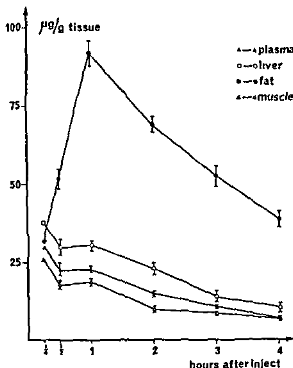


Fig 2 Distribution of enibomal (NFN) in rats after an intravenous injection of narko dorm® (60 mg enibomal sodium (NFN)/kg) Mean value ± S E M for 8 rats

each compound with the bile was therefore less than 2 % of the injected dose per hour The total excretion of each compound into the small intestine was also within the first four hours less than 2 % of the injected dose

Enibomal has a very great affinity to fatty tissue (fig 2) As early as 15 minutes after the injection the concentration was higher in the fat than in both the plasma and muscles The concentration in fat was seen to rise steeply during the first hour and thereafter fell again, but at a much slower rate Both muscle and liver displayed an abrupt fall in the enibomal concentration within the first 30 minutes This is followed however, by a minor rise during the period from 30 to 60 minutes after the injection After 60 minutes the concentration fell again, though at a slower rate than during the first 30 minutes of the experimental period

Due to interference of biogenic substances the concentration in the brain could not be determined in all cases However, the concentrations that could be determined averaged about 22 µg/g 15 minutes after the start of the experiment, falling to about 8 µg/g after one hour After four hours the concentration in the brain was about 6 µg/g

The plasma concentration of embomal (fig 2) showed a steep fall within the first 30 minutes, after which it remained at a constant level during the next 30 minutes The concentration then fell again at a rate corresponding to a half-value period for embomal of just under two hours

Discussion

Qualitatively the metabolism of embomal proceeds according to the same pattern in rats as observed in humans (RAVN JONSEN 1970), except that the rate is much faster It is evident from experiments on both the rat and the human that the process of demethylation to compound VIII requires a certain latency The oxidation to VI, on the other hand, starts very soon

WELLES *et al* (1963) found that 50 per cent of a given dose of methohexital (enallynymal NFN) is excreted with the bile within the first hour and a total of 83 per cent is excreted with the faeces in the course of 48 hours This is excreted mainly in the form of 5-hydroxy methohexital Other barbiturates are also excreted with the bile but in smaller amounts (FREY 1959b, KLAASSEN 1971) Analyses of the bile and of the small-intestinal contents reveals no similar excretion of embomal or metabolites within the first four hours after the injection The excretion into the intestinal is without or of little importance in the elimination of embomal

FREY'S (1959a) observation that the sleeping period was the same for fat and lean rats after the administration of equal doses of embomal per kilogram cannot be explained by a decreased affinity of embomal to fatty tissue Our investigation showed that within the first hour after the injection of embomal a large proportion was deposited in the fatty tissue (fig 2) The analytical method used ensured that the substance deposited was actually embomal Qualitatively the depositing displayed great resemblance to that demonstrated for thiopental (thiomebumal NFN) SHIDEMAN *et al* (1953), for instance, also found a maximum concentration of thiopental in the fatty tissue of rats about one hour after its administration Quantitatively, on the other hand, differences are seen While SHIDEMAN *et al* (1953), after the administration of 30 mg thiopental per kilogram, found a maximum concentration of about 140 μg/g, we found the maximum concentration of embomal to be only 90 μg/g after the injection of 60 mg/kg

No satisfactory explanation can be given as to why the plasma concentration remains constant during the period from 30 to 60 minutes after the injection The possibility should be considered that the distribution in the more blood perfused organs is completed within the first 30 minutes, and that the metabolism does not become of significant importance until after one hour

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REFERENCES

Frey H H Vergleichende Untersuchungen zum Stoffwechsel Intravenoser Kurznarkotika *Arch Int Pharmacodyn* 1959a 118 12–61

Frey H H H Kleinebrand & D Krause Untersuchungen über d e Stoffwechselleistung der Leber beim Barbiturat Abbau *Arch Int Pharmacodyn* 1959b 121, 125 133

Klaassen C D Biliary excretion of barbiturates *Brit J Pharmacol* 1971, 43 161 166

Ravn Jonsen A The urinary excretion of enibomalum (NFN) in man after adm nistration of anaesthetic doses *Acta pharmacol et toxicol 1970 28 484–492.*

Shideman F E T C Gould W D Winters R C Peterson & W K Wilner The distribution and *in vivo* rats of metabol sm of thiopental *J Pharmacol Exp Therap* 1953 107 368–378

Welles J S R E McMahon & W J Doran The metabolism and excretion of methohexital in rat and dog *J Pharmacol Exp Therap* 1963 139 166–171

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Teratogenic and Embryotoxic Effects of the Herbicides Di- and Trichlorophenoxyacetic Acids (2, 4-D and 2, 4, 5-T)

By

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Abstract Commercial solutions of phenoxyacetic acids were tested for teratogenic effects in NMRI mice The Swedish product Hormoslyr 500 T contained only 2,4,5 trichlorophenoxyacetic acid (2,4,5 T) while Hormoslyr 64 was a mixture of 2,4 dichlorophenoxyacetic acid (2,4-D) and 2,4,5 T (2 1) Subcutaneous injections were given from day 6 through day 14 of pregnancy and the animals were sacrificed on day 18 The number of resorbed embryos, living embryos with gross malformations, as well as internal and skeletal malformations were recorded It was found that both preparations at the high dosage (110 mg/kg/day) were teratogenic and embryotoxic At the low dose level (50 mg/kg/day) the 2,4,5-T solution was more harmful than the mixture of 2,4 D and 2,4,5-T The risks of teratogenicity in human civilian use and the role of dioxins are discussed

Key words Teratogenesis – embryotoxicity – herbicides – 2,4-D – 2,4,5 T

The lively discussion of the possible hazard to man of the use of large quantities of herbicides in agriculture and forestry has been focused upon the chlorinated phenoxyacetic acids, even though these compounds have been considered to be only moderately toxic in animals (Rowe & Hymas 1954) The report by Courtney *et al* (1970) on teratogenic and embryotoxic effects of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and to some extent of 2,4-dichlorophenoxyacetic acid (2,4-D) in experimental animals was the basis for the rapidly growing interest in the various adverse effects of these herbicides The most significant malformations induced at high doses are cleft palate in mice and cystic kidneys in rats and mice That the teratogenic effect is due not only to the 2,4,5-T but also to the high content (27 p p m) of the dioxin contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was confirmed in a later study (Courtney & Moore 1971)

Several studies on 2,4,5-T, 2,4-D and dioxins have since been performed

in different species and these essentially confirm that the two phenoxyacetic acids at high doses are teratogenic and embryotoxic and show that TCDD is teratogenic in the mouse (ROLL 1971, NEUBERT & DILLMANN 1972) It is interesting to note that even with high doses (40 mg/kg 2,4,5-T and 80 mg/kg 2,4-D), it has not been possible to produce malformations in monkeys (WILSON 1972a) However, two out of four females aborted after 2,4-D treatment (WILSON 1972b) The report that phenoxyacetic acids cause abortions in reindeers in Sweden was later not confirmed in a study by EHRNE (personal communication) Several newspaper reports of human malformations in Sweden, Vietnam and Arizona which had been attributed to the mothers' alleged exposure to the phenoxyacetic acids have been evaluated on more than one occation but it has not been possible to point at a causal relationship (LARSSON 1971)

2,4,5-T and 2,4-D as different esters, salts or acids have been commonly used as weed killers for more than 20 years In Vietnam they have been used as defoliants and crop-destroyers in quantities 10–15 times that recommended for civilian use The regulations for use in the U S A and Sweden have recently been restricted For example, the use of phenoxyacetic acids in forestry was temporarily banned in March, 1971, by the Swedish Poisons and Pesticides Board in Sweden The case was to be opened for re-evaluation at the beginning of 1972 when additional data would be available Most of the reports so far have dealt with studies on 2 4 5 T with various dioxin contents However, since the phenoxyacetic acids used as herbicides for civilian purposes usually contain both 2,4,5-T and 2 4 D together with emulsifiers and solvents, and since mechanisms of interaction, e g synergism, cannot be ruled out untried, it has been considered to be of practical interest to test the teratogenic effects of commercial samples In the following study the effects on mice of two commercial phenoxyacid formulations in Sweden, Hormoslyr 64 (2,4 D and 2 4,5-T, 2 1) and Hormoslyr 500-T (2 4 5-T) have been tested in a double blind test

Material and Methods

Three-month old random bred NMRI mice (obtained from Gl Bomholtgaard DK 86 Ry Denmark) were used The animals were kept in macrolone cages in a room with constant temperature (23°) and automatically regulated light from 6 a m to 6 p m and fed standard lab chow (Ewos Sodertalje Sweden) and water *ad libitum* The females were mated overnight and checked for vaginal plugs the next morning denoted as day zero of pregnancy The females were weighed on this day and also from days 6 to 18

The test solutions were prepared and coded for the double blind test at the Swedish Poisons and Pesticides Board The substances (kindly supported from Gullviks Fabriks

Table 1

Main teratogenic and *embryotoxic* effect on NMRI mice after treatment with 2 4 5 T or 2 4 D/2 4 5 T (2 1) from day 6 through day 14 of gestation

Group	Daily treatment Substance	Dose s c mg/kg	No of litters	No of implantation sites	% of resorbed implantation sites	No of living foetuses	No of foetuses with cleft palate	% of foetuses with cleft palate	Mean weight of living foetuses g	± S D
A	2 4 5 T	110	18	203	70 4	60	44	73 3	0 76	± 0 14
B	2 4 5 T	50	19	247	23 1	190	38	20 0	0 99	± 0 16
C	2 4-D+2 4 5 T	110	20	250	55 6	111	75	67 6	0 78	± 0 14
D	2 4-D+2 4 5 T	50	20	256	14 5	219	21	9 6	1 05	± 0 14
E	Vehicle	0	19	218	12 4	191	9	4 7	1 07	± 0 14

AB Malmo, Sweden) used for preparing the solutions were the commercially available Hormoslyr 500-T which contains the butoxyetylester of 2,4,5 trichlorophenoxyacetic acid (500 g/l), and Hormoslyr 64 which contains 2 4 dichlorophenoxyacetic acid (330 g/l) and 2 4 5 trichlorophenoxyacetic acid (170 g/l) The content of dioxins, mainly TCDD, was less than 1 p p m and the most common dioxin was 2 3 7 8 tetrachlorodibenzo-p dioxin (verified at the Swedish Poisons and Pesticides Board) Dimethylsulphoxide (DMSO) was used as a vehicle of the test solutions, which also contained 19 mg/ml of the solvent (a non specified mixture of petroleum distillates) The animals were given subcutaneous injections 0 1 ml/20 g body weight at 10 a m from day 6 through day 14 of gestation The daily dose of phenoxyacetic acid for the different groups was as follows

A 110 mg/kg of 2,4 5 T
B 50 mg/kg of 2,4 5 T
C 110 mg/kg of 2,4-D/2 4,5 T (2 1)
D 50 mg/kg of 2,4-D/2,4 5 T (2 1)
E 0 mg/kg controls given 0 1 ml/20 g of the vehicle)

The number of animals treated in the five experimental groups are given in table 1 None of the treated dams died or showed any signs of adverse effects except for local necroses at the injection sites which appeared after a few days

On day 18 of gestation the animals were sacrificed by cervical dislocation The living foetuses were removed weighed and examined for gross malformations The number and size of the dead and resorbed foetuses were noted One third of the living foetuses from each litter was fixed in ethanol, cleared in potassium hydroxide and stained with Alizarin red S for examination of skeletal malformations (DAWSON 1926–1928) The remainder of the living foetuses was fixed in Bouin s solution for subsequent sectioning and examination for internal malformations (WILSON 1965)

Results

Foetal mortality Foetal mortality was significantly increased at both dose levels of 2,4,5 T and at the high dose of the 2,4-D/2,4,5-T mixture, see table 1 The most severe effect was obtained in the two groups given the highest phenoxyacid dose, see table 2 Thus, every litter in group A and C showed resorptions and 33 % and 25 % of the litters respectively were totally resorbed In group B only 2 of 19 litters showed more than 50 % resorption Group D showed more litters with resorptions than the control group E, even if the resorption rate was almost similar in these groups

Isolated cleft palate An increased frequency of cleft palate was also observed in the same groups and to the same degree as described for the incidence of foetal mortality, see table 1 Litters with cleft palate in all surviving foetuses were obtained only in the high dose groups in A, 6 out of 12 litters and in C, 6 out of 15 litters

Retarded foetal growth The foetal weight was considerably reduced in animals treated with the high doses of the phenoxyacetic acids while no definite effect was registered after treatment with the low doses (table 1)

Table 2.

Distribution of resorption frequency in litters from NMRI mice after treatment with 2,4,5 T and 2,4-D/2,4,5 T (2 1) from day 6 through day 14 of gestation

Group	Daily treatment Sub- stance	Dose s c mg/kg	No of *litters*	Litters with at least one foetus resorbed no	%	Litters with > 50 % resorbed foetuses no	%	Litters with all resorbed foetuses %	no
A	2,4,5 T	110	18	18	100	13	72	6	33
B	2,4,5 T	50	19	16	84	2	4	0	
C	2,4 D+								
	2,4,5-T	110	20	20	100	12	60	5	25
D	2,4-D+								
	2,4,5 T	50	20	15	75	0		0	
E	Vehicle		19	11	58	0		0	

The slightly low-weight foetuses most often showed retarded skeletal development and reduced amounts of brown fat in the cervical region Very few of the foetuses in the control group showed these signs of retarded development.

Skeletal malformations After the highest doses of phenoxyacetic acids the percentage of foetuses with malformations of ribs and vertebrae was double that of the other three groups, including the controls (table 3) In addition, the degree of malformations was much more severe in these two groups, especially in group A

Table 3

Skeletal and internal malformations in NMRI mice after treatment with 2,4,5 T and 2,4 D/2,4,5 T (2 1) from day 6 through day 14 of gestation

Group	Daily treatment Sub stance	Dose s c mg/kg	Rib and vertebral malformations no *	%	Haemorrhages subcutaneous no	%	renal no	%	Dilated *renal pelvis* no	%
A	2,4,5 T	110	6/24	25 0	10/60	16 7	1/36	2 8	3/36	8 3
B	2,4,5 T	50	7/70	10 0	9/190	4 7	0/120		4/120	3 3
C	2 4-D+									
	2,4,5 T	110	11/44	25 0	21/111	18 9	11/67	16 4	5/67	7 5
D	2,4-D+									
	2 4,5 T	50	7/82	8 5	6/219	2 7	6/137	4 4	4/137	2 9
E	Vehicle		8/68	11 8	10/191	5.2	3/123	2 4	7/123	5 7

* No with malformations/number examined

Table 4

Examples of spontaneously occurring malformations in NMRI mice after treatment with 2 4 5 T and 2,4 D/2 4 5 T (2 1) from day 6 through day 14 of gestation

Group	Daily treatment Sub-stance	Dose s c mg/kg	No of living foetuses	No of foetuses with exencephaly	hydro-cephaly	open eye	micro-gnatia	ectro dactyly
A	2 4 5 T	110	60	2	1	1	3	1
B	2 4 5 T	50	190	3	1	2	–	–
C	2 4 D+ 2 4 5 T	110	111	–	4	1	3	1
D	2,4 D+ 2 4 5 T	50	219	2	1	–	–	–
E	Vehicle		191	2	1	1	1	–

Haemorrhages Subcutaneous bleeding with a large variation in location was found to be three times as common in the two high dose groups as in the other three groups (table 3) The frequency of renal haemorrhage was increased only in the group given 2,4 D/2,4,5-T in the high dose (table 3) No gastro-intestinal haemorrhages were detected

Renal malformations Cystic kidneys were not found Foetuses with different degrees of dilated renal pelves were found of which only the more pronounced ones were recorded (table 3) Only a slightly increased frequency was observed in the high dose groups The foetal kidneys in the two high dose groups usually appeared smaller and opaque in the sections as compared with the controls which were larger and had translucent medullae

Spontaneous malformations In the investigated material a few malformations appeared in all the groups as shown in table 4

Discussion

The present investigation confirms earlier studies, which have shown that high doses of phenoxyacetic acids are teratogenic, cause foetal death and retarded foetal growth in mice (COURTNEY *et al* 1970, ROLL 1971, NEUBERT & DILLMANN 1972) The substances tested which are used as weed killers in Sweden do not, however, indicate any greater teratogenic risk than 2,4 5-T or 2,4-D compounds used in other experimental studies

The various types of malformations found in this study were identical with those reported by other investigators The most common defect was

isolated cleft palate Cystic kidneys which was one of the most characteristic malformations observed by COURTNEY *et al* (1970) after 2 4 5 T treatment were not found Nor did we observe any marked dilatation of the renal pelves Less frequent, but probably also caused by the treatment, were skeletal malformations and subcutaneous haemorrhages A frequent observation was that the skin of the living foetuses, especially the small ones was very sensitive to handling with easily occurring subcutaneous bleeding The phenomenon was mainly observed after treatment with the phenoxyacetic acid mixture at the higher dose but it also occurred in the other groups Handling NMRI foetuses of the same age from untreated litters (unpublished observation) did not cause this type of subcutaneous bleeding It cannot be ruled out that the dimethylsulphoxide or the trace quantities (less than 1 p p m) of dioxin present caused some of the subcutaneous bleeding (CAUJOLLE *et al* 1967) Gastro intestinal bleeding was not detected though it has been reported to occur after 2,4,5 T treatment or dioxin treatment (COLLINS & WILLIAMS 1971, COURTNEY *et al* 1971, SPARSCHU *et al* 1971) Only the mixture of 2 4 D/2,4,5-T at the 110 mg/kg dose increased the frequency of renal haemorrhages

Earlier studies indicated that 2,4,5 T was more teratogenic than 2 4 D (MRAK 1969) It is interesting to note that the foetal damaging effect of the combination of 2,4-D/2,4,5 T did not exceed the effect of 2,4 5-T at a comparable dose of phenoxyacetic acid Concerning the teratogenic effects of the two substances in combination at 110 mg/kg it seems however, as if the 2,4 D contributes more than would be expected from purely additive effect Since only few studies on 2,4 D derivatives are available the results indicate that further studies should be carried out to investigate a possible synergistic effect of the two phenoxyacetic acids

LINDQUIST & ULLBERG (1971) suggested an interference with embryonic nutrition as a possible mechanism for the teratogenic action of the herbicides 2,4 5 T and 2 4 D In spite of a rather even distribution on whole body autoradiographs of the two substances there was a much more rapid disappearance from all tissues for 2,4 D and a stronger accumulation in the yolk sac placenta for 2 4,5-T (LINDQUIST & ULLBERG 1971) The selective uptake of 2,4 5 T in the yolk sac placenta and a lack of placental transfer in early gestation was interpreted in favour of a similar teratogenic mode of action for 2,4,5 T at that postulated for trypan blue (LLOYD & BECK 1969) However, an inhibition of the embryonic nutrition seems to be a less likely cause for, e g cleft palate since even single injections of 2,4 5 T on day 13 (NEUBERT & DILLMANN 1972) can cause cleft palate and at that stage the main nutritional role of the yolk sac placenta is over

In all animal experiments the dose resulting in teratogenic effects has been extremely high as compared with the dose which a pregnant woman could

normally be exposed to Thus, the results of the present and other studies do not substantiate any special risk to the human embryo from the regular use of phenoxyherbicides

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REFERENCES

Caujolle, F M E, D H Caujolle, S B Cros & M-M J Calvet Limits of toxic and teratogenic tolerance of dimethyl sulfoxide *Ann N Y Acad Sci* 1967, 141, 110–126

Collins, T F. X. & C. H Williams Teratogenic studies with 2,4,5 T and 2,4-D in the hamster *Bull Environ Contam Toxicol* 1971, 6 559–567

Courtney, K D, D W Gaylor, M D Hogan, H L Falk, R R Bates & J Mitchell Teratogenic evaluation of 2 4,5 T *Science* 1970, 168, 864–866

Courtney, K D & J A Moore Teratology studies with 2,4,5 trichlorophenoxyacetic acid and 2,3,7,8 tetrachlorodibenzo-*p*-dioxin *Toxicol appl Pharmacol* 1971, 20, 396–403

Dawson, A. B A note on the staining of the skeleton of cleared specimens with Alizarin Red S *Stain Technol* 1926–28, 1, 123

Larsson, K S. Yttrande över fenoxisyror In *Fenoxisyror Granskning av aktuell information Rapport från en expertgrupp till Giftnamnden* AB Allmänna forlaget, Stockholm, 1971

Lindquist, N G & S Ullberg Distribution of the herbicides 2 4 5 T and 2,4-D in pregnant mice Accumulation in the yolk sac epithelium *Experientia* 1971, 27, 1439–1441

Lloyd, J B & F Beck Lysosomes and congenital malformations *Biochem J* 1969, 115, 32–34

Mrak, E M Report of the Secretary s Commission on Pesticides and Their Relationship to Environmental Health, 1969, p 666 Department of Health, Education and Welfare, Government Printing Office, Washington, D C

Neubert, D & I. Dillmann Embryotoxic effects in mice treated with 2,4,5 trichlorophenoxyacetic acid and 2,3,7,8 tetrachlorodibenzo-*p* dioxin *Naunyn-Schmiedeberg's Arch Pharmacol* 1972, 272 243–264

Roll, R *Untersuchungen über die teratogene Wirkung von 2,4,5 T bei Mäusen Fd Cosmet Toxicol* 1971, 9 671–676

Rowe, V K. & T A Hymas Summary of toxicological information on 2 4 D and 2,4,5 T type herbicides and an evaluation of the hazards to livestock associated with their use *Amer J vet Res* 1954, 15 622–629

Sparschu, G L., F L Dunn, R. W Lisowe & V K Rowe Study of the effects of high levels of 2,4 5 trichlorophenoxyacetic acid on foetal development in the rat *Fd Cosmet Toxicol* 1971 9, 527–530

Wilson, J G Embryological considerations in teratology In *Teratology Principles and techniques* Ed J G Wilson and J Warkany The University of Chicago Press, Chicago and London 1965 p 251–277

Wilson J G Report on treatment of pregnant Rhesus monkeys with 2,4 D (2 4-di chlorophenoxyacetic acid) and CMPA (4 chloro 2 methylphenoxyacetic acid) to the Swedish Posisons and Pesticides Board 1972a

Wilson, J G Abnormalities of intrauterine development on non human primates In *The use of non human primates in research on human reproduction* WHO Symposium Ed E Diczfalusy and C. C. Standley WHO Research and Training Centre on Human Reproduction, Stockholm 1972b, 261–292

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Protein Binding of Drugs in Plasma from Patients with Acute Renal Failure

By

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Abstract It was found by *in vitro* experiments that the protein binding of acetylsalicylic acid, salicylic acid, phenylbutazone, diphenylhydantoin, sulphadiazine and thiopental was decreased in the plasma of 10 surgical patients with acute renal failure The plasma was taken 4 to 8 days after the onset of the renal failure At a drug concentration of 400 μg/ml the decreases ranged from 21 8 to 60.3 % as compared with a normal control group At drug concentrations in or near the normal therapeutic level the protein binding was even more decreased for the salicylic acids and sulphadiazine but less decreased for diphenylhydantoin and thiopental The decreased binding could only partly be explained by the lower concentration of albumin in the patients 3 0 g/100 ml ± 0 48 (S D) compared to 4 1 g/100 ml ± 0.53 (S D) (P < 0 01)

Key words Kidney failure – acute anuria – protein binding – drug therapy

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Acute renal failure (tubulo-interstitial nephritis (BRUN 1954)) is a complication which may occur in patients suffering from severe injuries Besides the renal changes the patients frequently show impaired hepatic function (cf BEECHER *et al* 1947, SHERLOCK 1963, THORÉN 1969) The concentration of serum proteins can be expected to be decreased (THORÉN 1969) Values for the serum albumin concentration in patients with acute renal failure are not given in the literature LANGE *et al* (1971) found in general agreement with other investigators that the average protein breakdown in 10 patients with acute renal failure was 105 g/24 hrs during the first eight days

KUNIN (1967) and BENNETT *et al* (1970) have given practical guides to drug usage in patients with impaired renal function and HEDGER (1971) gives a similar guide for patients suffering from acute renal failure Apparently drug therapy in these patients is usually based on knowledge of the biological half-life of the drug in normal subjects and on the creatinine clearance of the patients (cf BERNE & BARBOUR 1971)

The purpose of the *in vitro* experiments reported here was to elucidate the following questions

1 Is the drug binding capacity of the plasma proteins affected in surgical patients with acute renal failure?

2 Is the concentration of drug binding protein reduced?

3 Is the drug binding capacity of the individual protein molecules changed?

Materials and Methods

Patients The albumin concentration in plasma was determined in 27 surgical patients suffering from acute renal failure The first 10 patients to be admitted to the haemodialysis unit were studied further The six men and four women were aged between 50 and 70 years and all suffered from severe injuries due to traffic accidents or complications of major surgery None of them had received drugs capable of interfering with the protein binding of the drugs investigated All the patients were seriously and acutely ill and confused and some were unconscious An accumulation of bilirubin was observed in three patients Very pronounced in patient 6 and less so in patients 1 and 2 It is noteworthy that patients 8 and 9, the only patients among the ten, had an artificial piece of aorta implanted shortly before the onset of acute anuria

Plasma In all the plasma samples studied (including plasma from normal blood donors) heparin was used as the only anticoagulant All the samples had been kept deepfrozen for some weeks before use The plasma from the patients was prepared from blood left in the dialyser (a KOLFF twin coil KIDNEY) following a regular therapeutic haemodialysis performed between 4 and 8 days after the onset of the renal failure

Drugs The protein binding of six drugs was investigated *Salicylic acid* (pK_A 3 0) was assayed as described by TRINDER (1954) *Acetylsalicylic acid* (pK_A 8 3) was also assayed by TRINDER s method after the addition of an equal volume of 1 N NaOH *Diphenylhydantoin* (pK_A 8 3) was determined by the method of SVENSMARK *et al* (1960)

Sulphadiazine (pK_A 6 5) was assayed by the BRATTON & MARSHALL method (1939) *Phenylbutazone* (pK_A 4 4) was determined as follows A 0 5 ml sample with 0 5 ml 1 N HCl is shaken for 2 minutes with 4 ml heptane After centrifugation 2.5 ml of the organic phase is transferred to a tube containing 1 ml 4 % Na_2CO_3 After shaking for another 2 minutes, the optical density in the carbonate phase is read at 265 nm The method yields a quantitative recovery from serum of 97 %

Thiopental (thiomebumalum NFN) (pK_A 7 6) Assay To a 0 5 ml sample is added 5 ml chloroform After shaking and centrifugation, 4 ml of the organic phase is transferred to a tube containing 1 ml 0 5 N NaOH After renewed shaking and centrifugation the optical density in the water phase is read at 305 nm The method yields a recovery of 83% from serum

In each experiment it was checked that the recovery of drug from the sample was the same as from a Krebs Henseleit solution (LOCKWOOD 1961) The blank values for the optical density were always quite low, except for acetylsalicylic acid where this value for donors as well as for patients was usually between 0 040 and 0 050 and the total reading between 0 300 and 0 400 *Albumin* Human albumin obtained from KABI was used The purity of the albumin was checked by electrophoresis *Determination of protein binding* The protein binding of the six drugs was deter

mined by an ultrafiltration technique which is a modification of the method described by ROLINSON & SUTHERLAND (1965) Lenghts of Visking cellophane tubing were well soaked in water, and bags were made by tying double knots on the tubing The water was wiped off, and the solution containing the drug was placed inside the bag which was then inserted into a vertical length of glass tubing (inner diameter 14 mm, tapered in the lower end) The upper end of the cellophane bag was connected to a cylinder containing compressed air, and a pressure of 1 kg/cm² was used to make an ultra filtrate of the solution The length of dialysing tubing beyond the knots was let out from the top of the glass tubing along with the stopper The first drops of ultra filtrate were always discarded The initial volume inside the bag was kept just sufficiently high to ensure that the volume reduction during the ultrafiltration did not exceed 10 % It was found that all six drugs during ultrafiltration of a protein free solution containing the drug, were present in the same concentration inside the bag and in the ultrafiltrate It was routinely checked that no protein penetrated the mem brane All experiments were done at room temperature

The calculations of protein binding were made on the assumption that the concen tration of the free drug inside the bag was the same as in the ultrafiltrate

The method described by RICHTERICH (1969) was used for the determination of total protein and the albumin concentration A urease method (slightly modified from CHANEY & MARBACH 1962) was used to assay the concentration of urea

Results

The plasma concentration of proteins in patients and in normal blood donors In table 1 are listed the average concentrations of total protein and of albumin in the plasma from surgical patients with acute renal failure The corresponding values for normal blood donors are also shown According to a calculation carried out as suggested by MANN & WHITNEY (1947) there is a statistically significant difference between the albumin concentrations in the two groups ($P < 0\ 01$) as well as between the total protein concentrations ($P < 0\ 05$)

Protein binding of drugs in patients and in normal blood donors Table 2 shows the binding of the six drugs to proteins in the plasma of 10 patients with acute renal failure and in plasma of five normal subjects (blood donors) In the table the binding is given as the percentage of bound drug

Table 1

Average concentrations of total protein and of albumin in the plasma from patients with acute renal failure and from normal blood donors Standard deviations are shown and n indicates the number of patients or the number of blood donors

	Patients	Normal blood donors
Total protein g/100 ml	5 9 ± 0 66 (n = 10)	6 6 ± 0 35 (n = 18)
Albumin g/100 ml	3 0 ± 0 48 (n = 27)	4 1 ± 0 53 (n [illegible]

Table 2

Protein binding of six drugs in the plasma of 10 patients with acute renal failure and five blood donors. Each value is the average of three experiments. Standard deviations are shown.

Donor	Percentage of bound drug					
	Salicylic acid	Acetyl salicylic acid	Phenyl butazone	Diphenyl-hydantoin	Thio-pental	Sulfa diazine
1	81.6	56.6	90.0	70.8	84.7	47.7
2	80.5	47.9	87.2	68.4	84.3	46.6
4	79.6	41.8	88.4	68.9	83.4	44.1
5	79.6	49.3	89.5	70.8	83.6	41.9
6	81.1	47.9	91.5	73.3	85.3	42.9
Average %	80.5 ± 0.9	48.7 ± 5.3	89.3 ± 1.6	70.4 ± 1.9	84.3 ± 0.8	44.6 ± 2.5
Average drug conc., μg/ml	442 ± 7.2	408 ± 14.8	500 ± 13.8	400 ± 29.8	433 ± 9.9	391 ± 11.8
Patient						
1	49.9	31.0	74.7	43.4	65.2	19.2
2	48.9	33.8	65.3	46.8	68.7	23.9
3	41.6	24.3	79.3	51.4	63.5	12.1
4	44.6	19.3	75.7	41.3	58.0	25.7
5	35.1	14.0	68.2	44.4	56.3	16.0
6	35.0	7.3	65.7	33.9	52.3	11.1
7	40.2	18.8	80.2	41.3	59.2	18.3
8	43.5	18.4	67.7	43.5	65.9	19.2
9	38.5	32.9	59.4	41.5	64.0	15.1
10	43.6	26.2	63.2	42.0	65.7	16.3
Average %	42.1 ± 5.1	22.6 ± 8.6	69.9 ± 7.1	43.0 ± 4.5	61.9 ± 5.2	17.7 ± 4.6
Average drug conc., μg/ml	396 ± 12.2	379 ± 9.6	461 ± 49.0	401 ± 21.1	430 ± 21.2	398 ± 11.5

Each value in the table is the average of three experiments. The standard deviation for each value has been calculated. For salicylic acid the standard deviations are between 0.1 and 2.5, for acetylsalicylic acid 0.3–2.8, for phenylbutazone 0.2–1.8, for diphenylhydantoin 0.2–4.5, for thiopental 0.2–1.5 and for sulphadiazine 0.3–2.4. The drug concentration was kept close to 400 μg/ml. The average values are also listed.

The average protein binding is lower in the patients than in the normal controls. The decrease in the percentual binding is most pronounced for sulphadiazine and for the two salicylic acids.

Table 3 shows the concentration of protein and of urea in the plasma samples listed in table 2.

Fig. 1 and 2 illustrate the binding of the six drugs to normal serum

Table 3

The concentration of protein and urea in the plasma of the patients listed in table 2 Standard deviations are shown.

Patient	Total protein g/100 ml	Albumin g/100 ml	Urea mg/100 ml
1	4 8	3 4	59
2	6 6	3.5	45
3	5 4	3.3	43
4	5 7	3 2	50
5	6 4	2 3	58
6	6 1	2.5	35
7	6 6	2 6	53
8	6 8	2 6	31
9	5.5	3 6	42
10	5 5	3 0	32
Average	5 9 ± 0 7	3 0 ± 0 5	45 ± 10 2

proteins Each single experiment is carried out in duplicate Fig 1 reveals that phenylbutazone brings about a very pronounced increase in albumin binding as the albumin concentration is increased from 0 to 1 % The drug concentrations were kept close to 400 µg/ml Sulphadiazine shows the lowest binding

Fig 2 shows the binding of the six drugs to proteins as a function of the total drug concentration Six blood donors were used and the plasma and plasma ultrafiltrate from each individual were used for each drug Human albumin was added to the ultrafiltrates and the albumin concentration was the same as in the plasma For all six drugs it appears as though the albumin molecules alone are very nearly able to bind the amount of drug bound in whole plasma

By using fig 1 and the average albumin concentrations for the donor group and for the patient group it is possible roughly to calculate how much the average amounts of drug bound per mg albumin could be expected to differ because of the lower albumin concentration in the patient group The results of this calculation are shown in table 4 Observed and calculated values for single experiments in six normal blood donors are also listed In the 10 patients the values observed are significantly lower than the calculated values This is true for all the six drugs, ($P < 0\ 01$ – the MANN-WHITNEY test) Sulphadiazine and the salicylic acids show the greatest difference between the observed and calculated values

Patient 6 had the greatest difference between the calculated and observed values in acetylsalicylic acid (76 %) Patient 6 also showed a relatively great

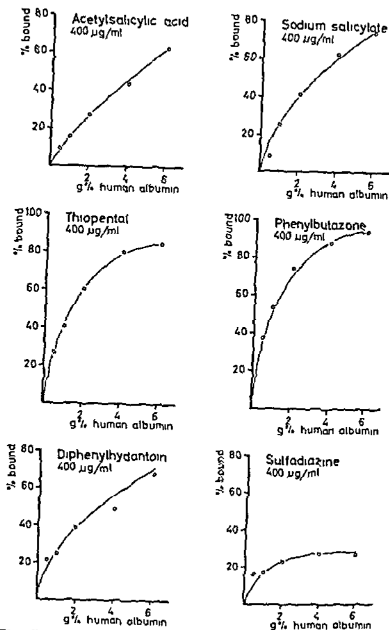


Fig 1 The albumin binding of six drugs as a function of the albumin concentration The solvent is a Krebs Henseleit solution (LOCKWOOD 1961) The drug concentration was kept as close as possible to 400 µg/ml

difference in the other drugs Patient 9 was the one who showed the greatest difference in salicylic acid (48 %), phenylbutazone (29 %) and diphenylhydantoin (37 %)

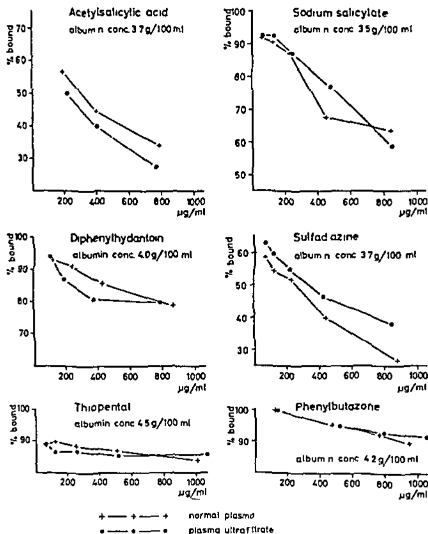


Fig 2 The binding to proteins of six drugs as a function of the total drug concentration Plasma and plasma ultrafiltrate from the same blood donor were used for each drug Human albumin was added to the ultrafiltrate so that the albumin concentration was the same as in the plasma Each value is the result of one experiment carried out in duplicate

Table 5 again shows the percentual binding of the six drugs to proteins in plasma from patients and from normal donors The drug concentrations are here kept as low as the accuracy of the drug assays allows As found with the high drug concentrations (table 2) the average binding is decreased in the plasma of patients For sulphadiazine and the salicylic acid the

Table 4

The average protein binding of six drugs in the plasma observed in 10 patients with acute renal failure The basic experimental data are the same as in table 2 Standard deviations are shown Observed and ' calculated"values for single experiments in six blood donors are also listed For further explanation see text

Amount of drug in µg, bound per mg albumin

	Salicylic acid	Acetyl salicylic acid	Phenyl butazone	Diphenyl hydantoin	Thio- pental	Sulpha diazine
Patients						
Value observed	5 6±0 6	2 8±0 8	10 9±1.5	5 9±1 1	9 0±1 1	2 4±0 6
"Calculated value	8 9±0 6	4 4±0 2	13.3±1 1	7 6±0 6	11 0±1 3	5.3±0 6
Difference in µg/mg	3 3	1 6	2 4	1 7	2 0	2 9
in %	37	36	18	22	18	55
Donors						
Value observed	7 9	4 6	9 7	8 6	8.9	4 4
' Calculated value	8 4	4 9	9 5	7 3	9 2	4 2

differences in percentual binding are greater at the low concentration than at the high concentration For phenylbutazone this difference is about the same for the two drug concentrations, and for thiopental and diphenylhydantoin the difference in binding appear to be smaller at the low concentration

Discussion

The binding to plasma proteins of six commonly used drugs was investigated in therapeutically dialysed plasma from patients with acute renal failure This plasma was the only one available in a large volume, and it should be emphasized that it is more effectively dialysed than the plasma obtained direct from a patient immediately on dialysis The urea concentration was 39 to 59 mg/100 ml, while it was between 100 and 150 mg/100 ml in the blood sample withdrawn from patients immediately on dialysis

The concentration of total plasma proteins was low in our surgical patients with acute renal failure and the decrease was exclusively due to the albumin fraction (table 1 and 3)

Table 5

Protein binding of six drugs in therapeutically dialysed plasma of surgical patients suffering from acute renal failure. 50, 100 of 200 µg drug was added pr ml plasma Protein binding in normal plasma (of blood donors) is also shown Standard deviations are indicated

		Salicylic acid	Acetyl salicylic acid	Phenylbutazone		Diphenyl hydantoin	Thiopental		Sulphadiazine	
Blood donors (each drug investigated in three donors)	Average drug conc µg/ml	116 ± 6 6	96 ± 6 9	–	233 ± 11 4	123 ± 9.3	55 ± 9 5	109 ± 20 7	58 ± 2 8	115 ± 5 3
	Average per cent bound	98 9 ± 1 6	57 3 ± 7 1	–	98 3 ± 0 6	92 5 ± 2 8	88 7 ± 0 4	89 1 ± 0 6	59 5 ± 1.2	57 1 ± 2 9
Patients (each drug investigated in three patients)	Average drug conc µg/ml	97 ± 10 2	97 ± 8 3	117 ± 3 0	230 ± 8 7	114 ± 6 2	51 ± 5 6	101 ± 11.2	51 ± 5.5	105 ± 10 1
	Average per cent bound	38 5 ± 3 3	11 8 ± 12 0	88 8 ± 5 7	78 2 ± 7 6	75 4 ± 10 9	70 3 ± 14 1	68.2 ± 14 0	20 8 ± 13 4	19 5 ± 12.3

The experiments reported in fig 2 shows that the albumin fraction in normal plasma is able to bind the same or almost the same amount of drug as that which is bound by all plasma proteins

Many investigators have described the protein binding of drugs Excellent review articles have been written by GOLDSTEIN (1949) and by MEYER & GUTTMAN (1968) MEYER & GUTTMAN emphasize that much evidence has been accumulated demonstrating that plasma protein binding can influence the distributional, pharmacological and pharmacokinetic properties of certain drugs

Salicylates REYNOLDS & CLUFF (1960) have found that salicylate is bound only to plasma albumin They also found a decreased binding of salicylate in the serum of patients with acute infections HAWKINS *et al* (1969) have investigated structural changes in human serum albumin induced by the ingestion of acetylsalicylic acid They showed that a trans acetylation reaction occurs *in vivo* between aspirin and human albumin

Diphenylhydantoin LUNDE *et al* (1970) found that at a concentration of 16 μg/ml, 93 % of diphenylhydantoin was bound in normal human plasma. REIDENBERG *et al* (1971) found a decreased binding of diphenylhydantoin in chronically ill patients with poor renal function The authors found normal binding of the basic drug desmethylimipramine in the uraemic patients A change in the plasma proteins was suggested as the explanation for the decreased binding

Sulphadiazine ANTON published several studies on the very variable protein binding of different sulphonamides – always using an equilibrium dialysis method (ANTON 1960) ANTON & COREY (1971) reported a decreased binding of sulphamethazine in six nephrectomized patients The mean period of time since nephrectomy was 17 months Buttner *et al* (1964) found a lower protein binding of sulphamoxol and sulphisomidin (sul faisodimidinum NFN) in patients with renal disease The decrease could not solely be explained by the lower albumin concentrations A comparison between the values for protein binding of sulphadiazine in normal plasma mentioned by ANTON & COREY (1971) with values observed by KRUGER THIEMER *et al* (1965) and also observed in the present study suggests that the percentual binding may be found to be slightly higher if ultrafiltration is used instead of equilibrium dialysis

Thiopental BRODIE *et al* (1950) demonstrated that at plasma levels of 10–50 μg/ml 75 % was bound to the non diffusible component of plasma GOLDBAUM & SMITH (1954) found that increasing the concentration of bovine albumin from 0 1 % to 6 % increased the bound thiopental from approximately 34 % to 90 % TAYLOR *et al* (1954) found a protein binding of 60 % with a thiopental concentration of 18 μg/ml in a solution containing 2 17 g human albumin per 100 ml It is concluded that no or almost none

of the drug is bound to proteins other than albumin The authors also studied the plasma protein binding of thiopental in normal and nephrectomized rabbits In *in vitro* experiments (18 μg thiopental per ml) they found a reduction from 75 % to 65 % in the binding to plasma protein, twenty four hours after nephrectomy Only in 2 of 4 experiments could they explain the decreased binding by a reduction in the albumin concentration

Phenylbutazone Since the studies of BURNS *et al* (1953 & 1955) phenylbutazone at plasma concentrations of about 100 μg/ml, has been known to be extensively (98 %–99 %) bound to proteins in human plasma

Table 2 shows that the percentual protein binding of all the six drugs (at very high drug concentrations) is decreased in patients with acute renal failure Table 5 indicates that the same is true at drug concentrations in the therapeutic range (only diphenylhydantoin has not been investigated at a 'clinical level") It is interesting that the decreases for the salicylic acids and sulphadiazine seem more pronounced at the low (therapeutic) concentration than at the high concentration The opposite is true for diphenylhydantoin and thiopental

There are three possible explanations for the decreased binding

1 A lower concentration of protein in the patient plasma

2 "Damaged' or incompletely synthetized proteins

3 Accumulation of compounds which also have affinity for the drug binding sites on the proteins

The importance of possibility number one has been elucidated by a calculation of μg drug bound per mg albumin There is no linear relationship between the albumin concentration and the drug binding capacity of the albumin Therefore fig 1 had to be used to calculate the amount of albumin binding that could be expected to change because of the lower albumin concentration in the patient group as compared with the normal group Judging from table 4, the decrease in protein binding cannot be due to the lower albumin concentration only This is particularly true for sulphadiazine and the salicylic acids It is concluded that the decrease in protein binding is partly but not entirely due to the lower albumin concentration

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REFERENCES

Anton A H The relation between the binding of sulfonamides to albumin and their antibacterial efficacy *J Pharmacol exp Therap* 1960 129 282–290

Anton, A H & W. T Corey Interindividual differences in the protein binding of sulfonamides The effect of disease and drugs *Acta pharmacol et toxicol* 1971, **29**, suppl 3, 134–151

Beecher, H K, F A Simeone, C H Burnett, S L Shapiro, E R Sullivan & T B Mallory The internal state of the severely wounded man on entry to the most forward hospital *Surgery* 1947, **22**, 672–711

Bennett, W M, I Singer & C H Coggins A practical guide to drug usage in adult patients with impaired renal function *J Amer med Ass* 1970, **214**, 1468–1475

Berne, T V & B H Barbour Acute renal failure in general surgical patients *Arch Surg* 1971, **102**, 594–597

Bratton, A C & E K. Marshall A new coupling component for sulfanilamide determination *J biol Chem* 1939, **128**, 537–550

Brodie, B B, L C. Mark, E M Papper, P A Lief, E Bernstein & E A Rovenstine The fate of thiopental in man and a method for its estimation in biological material *J Pharmacol exp Therap* 1950, **98**, 85–96

Brun C *Acute anuria* Munksgaard, København 1954

Burns J J, R K Rose, T Chenkin, A Goldman, A Schulert & B B Brodie The physiological disposition of phenylbutazone (butazolidin) in man and a method for its estimation in biological material *J Pharmacol exp Therap* 1953, **109**, 346–357

Burns, J J, R K Rose, S Goodwin, J Reichenthal, E C. Horning & B B Brodie The metabolic fate of phenylbutazone (butazolidin) in man *J Pharmacol exp Therap* 1955, **113**, 481–489

Buttner, H, F Portwich, E Manzke & N Staudt Zur Pharmakokinetik von Sulfonamiden unter pathologischen Bedingungen *Klin Wschr* 1964, **42**, 103–108

Chaney, A L & E P Marbach Modified reagents for determination of urea and ammonia *Clin Chem* 1962, **8**, 130–132

Goldbaum, L. R & P K Smith The interaction of barbiturates with serum albumin and its possible relation to their disposition and pharmacological actions *J Pharm exp Therap* 1954, **111**, 197–209

Goldstein A The interactions of drugs and plasma proteins *Pharmacol Rev* 1949, **1**, 102–165

Hawkins, D, R N Pinckard, I P Crawford & R S Farr Structural changes in human serum albumin induced by ingestion of acetylsalicylic acid *J Clin Invest* 1969, **48**, 536–542.

Hedger, R W The conservative management of acute oliguric renal failure *Med Clin N Amer* 1971, **55** 121–135

Kruger Thiemer, E E Wempe & M Topfer Die antibakterielle Wirkung des nicht eiweissgebundenen Anteils der Sulfanilamide im menschlichen Plasmawasser *Arzneimittel Forsch* 1965, **15**, 1309–1317

Kunin, C M A guide to use of antibiotics in patients with renal disease *Ann Intern Med* 1967, **67**, 151–158

Lange, H, D Seybold & G Strohmeyer Eiweisskatabolismus und Freisetzung von Zellwasser bei akuten Nierenversagen *Dtsch med Wschr* 1971, **96**, 585–587

Lockwood, A P M "Ringer" solutions and some notes on the physiological basis of their ionic composition *Comp Biochem Physiol* 1961, **2**, 241–290

Lunde, P K M, A Rane, S J Yaffe, L. Lund & F Sjoqvist Plasma protein binding of diphenylhydantoin in man *Clin Pharmacol Therap* 1970, **11**, 846–855

Mann H B & D R Whitney On a test of whether one of two random variables is stochastically larger than the other *Annals of Mathematical statistics* 1947, 18, 50–60

Meyer, M C & D E Guttman The binding of drugs by plasma proteins *J pharm Sci* 1968, 57, 895–918

Reidenberg M M, I Odar Cederlof, C von Bahr, O Borgå & F Sjöqvist Protein binding of diphenylhydantoin and desmethylimipramine in plasma from patients with poor renal function *New Engl J med* 1971, 285 264–267

Reynolds, R C & L E Cluff Interaction of serum and sodium salicylate Changes during acute infection and its influence on pharmacological activity *Bull Johns Hopk Hosp* 1960 107, 278–290

Richterich, R. Serum protein by the biuret method In *Klinische Chemie* S Karger, Academic press, New York and London 1969 245–249

Rolinson, G N & R Sutherland The binding of antibiotics to serum proteins *Brit J Pharmacol* 1965, 25, 638–650

Sherlock S *Diseases of the liver and biliary system* Blackwell Sci Publ, Oxford 1963

Svensmark, O, P J Schiller & F Buchthal 5,5 Diphenylhydantoin (dilantin®) blood levels after oral or intravenous dosage in man *Acta pharmacol et toxicol* 1960, 16, 331–346

Taylor, J D, R K Richards J C Davin & J Asher Plasma binding of thiopental in the nephrectomized rabbit *J Pharmacol exp Therap* 1954 112, 40–48

Thorén, L *Vatskebalans* Almquist & Wiksell Stockholm 1969

Trinder, P Rapid determination of salicylate in biological fluids *Biochem J* 1954, 57, 301–303

Acta pharmacol et toxicol 1973, 32, 430–441

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The Effect of Mercaptodextran on Distribution and Toxicity of Mercury in Mice

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Abstract Mercaptodextran (SH dextran) proved to be less toxic than BAL in rodents and better tolerated in intravenous infusion therapy Immediate therapy with SH dextran was superior to any other treatment for the removal of sub lethal doses of mercury (1 8 μmol Hg/kg) from the body The life saving effect of SH dextran given immediately after a lethal $HgCl_2$ dose (74 μmol Hg/kg) was demonstrated When chelation therapy was given two hours after the poisoning however, SH dextran was without any significant effect, while BAL was still life-saving Mercury mobilized from the kidneys was redistributed to other organs when immediate chelation treatment with low molecular thiols was given, whereas SH dextran brought about an excretion of mobilized mercury SH dextran, in contrast to certain other antidotes did not induce an increased deposition of mercury in the brain

Key words Chelating agents – dextran derivatives – mercury poisoning – mice experiments

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It is generally accepted that most of the toxic effects of heavy metals are due to the blocking of vital SH groups of cellular proteins Antidotes to mercury and other heavy metals are assumed to exert their activity because of their ability to form stable complexes with the metal ions The most potent mercury detoxificating agents are SH containing compounds, of which D-penicillamine (β,β'-dimethylcysteine) (APOSHIAN 1958) and in particular BAL (2,3-dimercapto-1-propanol) (WATERS & STOCK 1945, GILMAN *et al* 1946, LONGCOPE & LUETSCHER 1946, LONGCOPE 1952, MASUI 1957) are used in clinical medicine

Unfortunately the practical usefulness of BAL is limited by its low therapeutic index (RANDALL & SEELER 1948, STOCKEN & THOMPSON 1949)

Efforts to develop less toxic thiols have resulted in compounds like N acetyl DL-penicillamine (APOSHIAN & APOSHIAN 1959) and 2-mercaptopropionylglycine (FUJIMURA *et al* 1964) In experimental and clinical tests however, these agents have failed to produce an effect comparable to that of BAL, and they are not recommended in acute mercury poisoning in recent reviews (BRUGSCH 1965, CHENOWETH 1968, CHISOLM 1970) The search for new chelating agents is therefore still fully justified

The present paper reports on the antidotal effect against mercury poisoning of a new macromolecular polythiol, viz mercaptodextran (SH-dextran) Its mercury complexing ability is comparable to that of BAL (JELLUM *et al* 1973) The present results demonstrate that SH dextran is less toxic than BAL in animals, and that immediate treatment with SH-dextran is the superior therapy in acute mercury poisoning

Materials and Methods

Materials

Dextrans with average molecular weight of 10 000 20 000 40 000 and 70 000 were purchased from Pharmacia AB Sweden and converted to SH dextrans (which will be denoted here SH 10 SH 20 etc respectively) according to the method of JELLUM *et al* (1973) 2 amino ethyl hydrogensulphate N acetyl homocysteine thiolactone 2 3 dimercapto-1 propanol (BAL) D penicillamine (PA) and sodium diethyl dithiocarbamate (DDC) were products of Koch Light Ltd England 2 mercaptopropionyl glycine (Thiola) was a gift from Santen Pharmaceutical Co Ltd Osaka Japan Radioactive mercuric chloride (^{203}Hg) was purchased from Radiochemical Centre Amersham England

Animals

White female mice (NMRI strain Gamle Bomholtgaard Laven Denmark) weighing about 20 g were used Food and water were available *ad libitum*

Mercury determinations

Radiomercury (^{203}Hg) content in whole animals was determined using a scintillation detector with a sodium fluoride crystal (model Intertechnique Selecteur type SA 40) In the centre of the crystal a cylindrical plastic vial (diameter 5 cm) was placed in which the standard samples in a total volume of 20 ml or the curled up dead animals were placed to be counted giving a constant counting efficiency of about 10 %

The radiomercury content of the separate mouse organs was determined using an automatic well counter (model Frieseke and Hoepfner) Kidneys liver and brain were dissected out and put into the standard counting tubes (diameter 1 5 cm) The setup had a constant counting efficiency of 25 % when specimens in a volume of 0 5 to 3 0 ml were counted

The radiomercury distribution in the electropherograms was assayed in a scintillation spectrometer after the strip had been cut into short pieces corresponding to the different species of serum protein

Results

Toxicity of mercaptodextran

Groups of six mice were given intravenously increasing volumes of 10 % or 25 % w/v solution of SH dextran in 0 1 M phosphate buffer at a pH adjusted to 7 4 before the injection The number of mice which died within 12 hours was noted

After the intravenous injection of 250, 375 and 500 μmol SH/kg of SH-10, SH-20, SH-40 and SH-70 no toxic effects were seen 625 and 825 μmol SH/kg (25 % solution of SH-20) given slowly intravenously, could be tolerated, whereas 1 0 mmol SH/kg immediately killed all the mice in a group of six animals Subsequent autopsy showed congested liver and caval veins, indicating that death had been caused by hypervolaemia. The colloid osmotic pressure of the plasma was increased by the injection of 10 % or 25 % of dextran-10,000, -20,000, -40,000 and -70,000 (HINT 1964), and the magnitude of the plasma expansion was assumed to be greater than the volumes of 10 % and 25 % SH dextran injected

A certain amount of dextran does not lead to the very considerable increase in blood volume when it is divided into several injections given throughout the day 1 8 mmol SH/kg of SH-10 and of SH-20 divided into six intravenous doses given at 4 hourly intervals did not give rise to any evidence of toxic effects

Mice kept on intravenous doses of 100 μmol/kg and 150 μmol/kg of SH-10 and of SH 20 per day for two weeks did not provide any evidence of toxic effects as far as weight, behaviour and appearance were concerned The kidneys and liver were found to be normal at histological examination

The results indicate that the toxicity of the SH groups in mercaptodextran is remarkably low However, the administration of large amounts of SH dextran involves the danger of fatal hypervolaemia

Effect of chelating agents on body distribution of mercury

After a single intravenous injection of mercuric chloride in rats the mercury is rapidly distributed to all organs, the kidneys being the major site of deposition (ROTHSTEIN & HAYES 1960) To determine the exact body distribution of mercury under the present conditions, all the test animals given chelation therapy, were parallelled by control animals For immediate or delayed chelation therapy the animals were given SH-10 or low molecular thiols

Solutions of mercuric chloride as well as of antidotes were prepared in a 0 1 M phosphate buffer with a pH adjusted to 7 4 The injected volume of toxic and of antidotal solutions was always 0 2 ml, and all injections were given intravenously The dose of mercury which was administered to

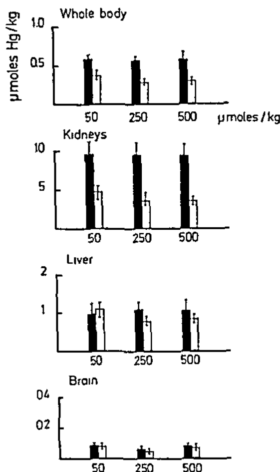


Fig 1 Effect of increasing doses of SH 10 on mercury distribution in mice SH 10 was given in doses of 50 250 and 500 µmol/kg respectively The Hg retention in the whole animal and in various organs 24 hrs after the intravenous injection of 1 8 µmol Hg/kg (as $^{203}HgCl_2$) is shown SH 10 was given as immediate intravenous treatment Each dose level was studied using six test animals parallelled by six controls The mean (of six) mercury content in the whole animal and in the separate organs is represented as columns ■ $HgCl_2$ alone □ $HgCl_2$ + SH 10 The bars indicate experimental range

all the mice both in the control groups and in the test groups, was 1 8 µmol/kg The preparation of mercury solution included the addition of radioactive mercuric chloride so as to obtain a specific activity of about 0 1 mci/mg Hg The injection solution was stored at 4°

A. *Effect of increasing doses of SH-dextran*

In fig 1 it is seen that 50 µmol SH/kg of SH 10 administered immediately

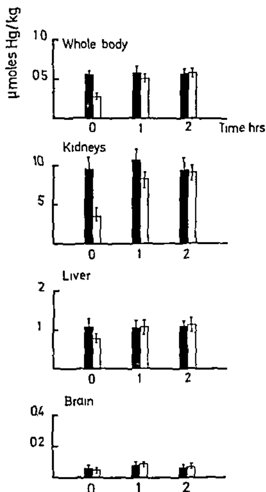


Fig 2 Effect of delayed treatment with SH 10 The influence of increasing intervals between the intravenous injection of mercury and of SH 10 (250 μmol SH/kg) on Hg retention in the whole body and in various organs in mice 24 hrs after the injection of 1 8 μmol Hg/kg (as $^{203}HgCl_2$) SH 10 was given immediately and 1 and 2 hours respectively after Hg injection Each time interval was studied using six test and six control mice ■ $HgCl_2$ alone □ $HgCl_2$ + SH 10 The bars indicate experimental range

after radiomercury reduced the amount of Hg retained in the whole body to about 65 % of the retention in the control animals 250 μmol SH/kg reduced the body burden to about 50 %, but higher doses did not result in an increased effect In particular the Hg-amount deposited in the kidneys was reduced by SH dextran The amount of Hg retained in the liver and brain was not significantly changed by SH-10

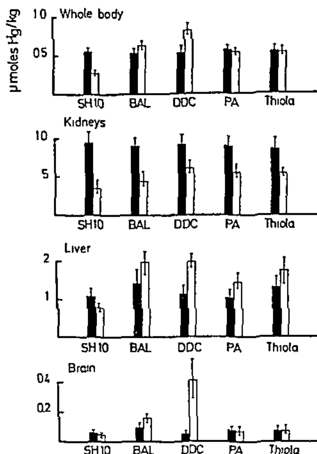


Fig 3 Effect of SH 10 compared to that of other chelating agents The influence of SH 10, BAL DDC PA and Thiola on Hg retention in the whole body and in various organs in mice 24 hrs after the intravenous injection of 1 8 µmol Hg/kg (as $^{203}HgCl_2$) The thiols were given as immediate intravenous treatment in a dose of 250 µmol SH/kg Each antidote was studied using six test and six control mice ■ $HgCl_2$ alone □ $HgCl_2$ + thiol The bars indicate experimental range

B *Effect of delayed treatment with SH dextran*

Fig 2 gives the amount of Hg retained in the whole body and in different organs when the interval between poisoning and treatment was 0, 1 and 2 hours respectively The beneficial effect of SH-dextran on Hg-retention in whole body and in kidneys decreased rapidly with increasing time intervals between poisoning and therapy, and the effect of SH-dextran was insignificant when treatment was delayed for two hours SH-10 had no significant effect on the Hg-content of the liver or brain whether it was given after 0, 1 or 2 hours

C *Effect of SH-dextran compared to that of other thiols*

Fig 3 shows the effect on Hg retention of immediate treatment with SH-10, BAL, DDC, PA and Thiola SH-dextran was the only agent which significantly reduced the burden of body mercury under these experimental conditions DDC significantly increased the body burden of mercury, whereas BAL, PA and Thiola had no significant effect on the amount of Hg retained in the whole body

All the agents used reduced the mercury deposition in the kidneys, but SH-dextran was the most active agent Thus all agents with the exception of SH-dextran brought about a redistribution of mercury in the body, i e the Hg content in organs other than the kidneys increased due to the treatment Therapy with DDC resulted in a more than fivefold increase in the amount of Hg deposited in the brain

Fig 4 shows the effect of SH dextran and BAL on Hg-poisoning when

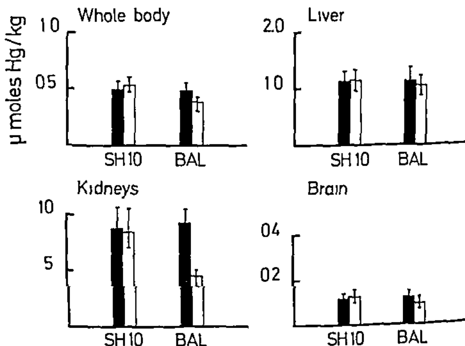


Fig 4 Effect of delayed treatment with BAL compared to that with SH 10 The influence of delayed treatment with SH 10 and BAL (dosage 250 μmol SH/kg) on Hg retention in the whole body and in various organs in mice 48 hrs after the intra venous injection of 1 8 μmol Hg/kg (as $^{203}HgCl_2$) The antidote was given intravenously 24 hrs after the mercury injection Each antidote was studied using six test and six control animals ■ $HgCl_2$ alone □ $HgCl_2$ + thiol The bars indicate experimental range

Table 1

EFFECT OF INTRAVENOUS TREATMENT WITH SH 10 AND BAL ON ACUTE MERCURY POISONING Mercuric chloride (74 μmol/kg) was injected intravenously Cumulative mortality within 14 days was registered The number of deaths/number of animals injected is given for the different groups of mice The amount of chelating agent given to mice in the test groups was 250 μmol SH/kg – Preliminary tests had showed that both 66 and 74 μmol Hg/kg killed 100 % of the mice in a group of six within 14 days whereas 55 μmol/kg killed 50 % of the animals

Mouse group	Treatment given immediately	Treatment given after 2 hours
Controls	6/6	6/6
SH 10-treated	0/6	6/6
BAL-treated	1/6	1/6

treatment was *delayed* for 24 hours SH-dextran had no significant effect, whereas BAL removed the Hg from the kidneys although the amount of Hg in the other organs was not significantly altered. In contrast to immediate therapy with BAL, the delayed BAL treatment resulted in a reduced total body burden of mercury

The lack of effect on delayed treatment with SH dextran is also seen in table 1, where the antidotes are given to mice poisoned with 74 μmol Hg/kg The table shows the cumulative mortality within 14 days of the poisoned controls and of test animals treated with SH 10 or BAL, immediately or 2 hours after the Hg injection Immediate treatment was life saving in both cases whereas SH dextran had no effect when treatment was delayed for 2 hours, although BAL was still life-saving

The ability of SH dextran to mobilize Hg from proteins in vitro

The excellent effect of immediate treatment with SH dextran in acute mercury poisoning was assumed to be due to its ability to form unusually stable complexes with mercuric ions (JELLUM *et al* 1973), an ability which may result in a removal of Hg fixed to serum and tissue proteins

The ability of SH dextran to detach Hg from serum proteins *in vitro* was studied by means of electrophoresis on cellulose acetate membranes of an incubated mixture of serum, $^{203}HgCl_2$ and SH 10 The incubation/separation process was carried out at pH 7 4 (phosphate buffer) and at pH 8 6 (barbitone buffer), which gave approximately the same results Fig 5 (pH 8 6) demonstrates the electrophoretic migration of the dextran mercaptide (top), and the electrophoretic pattern of mercaptides formed by incubation for 15 min of trace amounts of $^{203}HgCl_2$ with human serum (middle) When the latter (albumin concentration corresponding to about

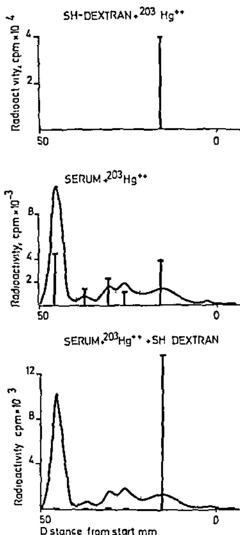


Fig 5 Effect of SH 10 on mercury bound to serum proteins The electrophoretic pattern of mercaptides formed by incubation for 15 min of trace amounts of $^{203}HgCl_2$ with SH 10 (top) and with human serum (middle) are shown When the latter is incubated with SH 10 (2 0 μmol/ml serum) for 15 min, a redistribution of radiomercury to SH 10 occurs (bottom) Continuous curve = protein concentration

0 4 μmol SH/ml) was incubated with SH-10 (2 0 μmol SH/ml) for 15 min at room temperature, a re-distribution of radiomercury to SH-10 occurred (bottom) *In vivo* a plasma concentration of 2 0 μmol SH/ml would result from intravenous therapy with a dose of about 120 μmol SH/kg if the dilution space of the thiol was limited to the blood plasma

The effect of SH-20 on Hg^{++} bound to proteins from different tissues is

Table 2

EFFECT OF SH 20 ON PROTEIN BOUND Hg^{2+}. Different organs were homogenized in distilled water (7 g tissue/100 ml) and incubated for 10 min with 10^{-4} M $^{203}HgCl_2$. One of two parallell homogenates from each tissue was treated with 2.5 10^{-3} M SH 20 (10 min incubation, room temperature). Protein in both parallells was precipitated by the addition of 10 % trichloroacetic acid in 1:3 volume to the solution, after which the solution was centrifuged in the cold. Protein bound mercury (as ^{203}Hg activity) was determined in a gamma spectrometer, both in the untreated and in the treated parallell. Protein from the latter was resuspended in 2.5 10^{-3} M SH 20, filtered off after 10 min., after which mercury determinations were repeated

Type of protein	Protein bound mercury (μmoles Hg/g protein remaining)		
	no treatment	SH 20 once	SH 20 twice
Liver	1.20	0.32	0.13
Kidney	1.16	0.25	0.11
Heart	1.21	0.21	0.12
Spleen	1.09	0.22	0.08
Serum	0.93	0.08	0.04

shown in table 2. The homogenized organs were incubated with $HgCl_2$ for 10 min. after which the proteins were treated twice with 10 % SH-20. Treatment once (table 2, middle column) detached most of the mercury fixed to the serum, spleen, heart, kidney and liver proteins, although protein SH from the kidney and the liver are known to complex Hg^{2+} very tightly. Still more Hg was picked up by SH-20 after a second treatment (right column). The trichloroacetic acid precipitation technique used here to determine protein bound mercury, is routinely used by other investigators (CLARKSON & MAGOS 1967). However, in the protein precipitate the pH decreases to between 1.0 and 2.0, which reduces the stability of mercaptides as shown by AASETH & KLAUSEN, (unpublished results).

Discussion

The practical usefulness of thiols has been limited by their intrinsic toxic effects. According to WATERS & STOCK (1945) the LD_{50} of BAL given intramuscularly to rats is 845 μmol BAL/kg. The toxicity of SH-dextran is remarkably low, as compared to other potent sulphur containing antidotes, thus 1.8 mmol SH/kg per day (divided into six intravenous injections) of SH-10 and SH-20 was given without any toxic effects. The fatal plasma expansion which was seen when a large amount of SH dextran was given as a single dose, was due to its high colloid-osmotic pressure.

With its high OH-content the Hg-chelate of SH-dextran is an extremely

hydrophilic macromolecule, which does not penetrate into the brain Presumably a significant chelate penetration across the blood brain barrier is restricted to nonpolar chelating molecules When DDC was given immediately after $HgCl_2$, a marked increase in Hg deposition in the brain resulted Immediate therapy with BAL also gave an increased brain uptake of mercury, confirming the report of MAGOS (1968) on rats, whereas delayed BAL treatment had no significant effect on the brain content of Hg

The dilution space of dextran is chiefly extracellular (GROTTE 1956) When treatment was delayed for two hours or more after Hg poisoning, the bulk of Hg had already passed intracellularly, and SH-10 therefore had no effect either on the body burden, distribution or toxicity of mercury PA is also known to have an almost exclusively extracellular distribution space, and it has no significant effect on the Hg distribution in rats when treatment is given 4 hours after $HgCl_2$ (AASETH & NORSETH, unpublished results)

When chelation therapy was given immediately after $HgCl_2$, all thiols produced a reduced deposition of Hg in the kidneys, but SH-dextran was the only agent which significantly lowered the total body burden of Hg The high ability of SH-10 to pick up Hg^{++} ions from serum proteins *in vitro* explains its good beneficial effect *in vivo* Furthermore the Hg-chelate of SH 10 is apparantly rapidly excreted, in contrast to chelates of low molecular thiols Although delayed treatment with BAL slightly decreased the total Hg retention in the body, immediate therapy with BAL as well as with PA and Thiola left the total body burden unchanged, and DDC even increased the amount of Hg retained Chelation therapy with PA, Thiola and DDC apparantly bring about the same re-distribution of Hg from the kidneys to other organs, which has been previously described in BAL therapy (ADAM 1951, NIGROVIC 1963, ULFVARSON 1963, BERLIN & LEWANDER 1965) This peculiar redistribution of Hg between the organs is not induced by Hg-chelation with mercaptodextran

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REFERENCES

Adam, K. R The effects of dithiols on the distribution of mercury in rabbits *Brit J Pharmacol* 1951, 6, 483–491

Aposhian, H V Protection by D penicillamine against the lethal effects of mercuric chloride *Science* 1958 128 93

Aposhian H V & M M Aposhian N acetyl DL-penicillamine a new oral protective agent against the lethal effects of mercuric chloride *J Pharm Toxicol* 1959, 126 131 135

Berlin M & T Lewander Increased brain uptake of mercury caused by 2,3 d mercaptopropanol (BAL) in mice given mercuric chloride *Acta pharmacol et toxicol* 1965 22 1–7

Brugsch H G Recent advances in chelation therapy *J Occup Med* 1965 7, 394–401

Chenoweth M B Clinical uses of metal binding drugs *Clin Pharmacol Therap* 1968 9 365 387

Chisolm J J Poisoning due to heavy metals *Pediatr Clin North Am* 1970 17, 591–615

Clarkson T W & L. Magos The effect of sodium maleate on the renal deposition and excretion of mercury *Brit J Pharmacol Chemother* 1967, 31, 560–567

Fujimura H T Akashi & T Hirasawa Pharmacological studies on thiol compounds (I) On the antidotal effect of α mercaptopropionylglycine (Thiola) *Folia phar macol jap* 1964 60 278–292.

Gilman A, R. P Allen F S Philips & E. S John Clinical uses of 2,3 dimercapto-propanol (BAL) X The treatment of acute systemic mercury poisoning in experimental animals with BAL, thiosorbitol and BAL glucoside *J clin Invest* 1946 25 549–556

Grotte, G Passage of dextran molecules across the blood lymph barrier *Acta Chir Scand* 1956 *Suppl* 211, pp 84

Hint H The relationship between the molecular weight of dextran and its effects *Rheomacrodex Reports of Symposia* Cardiff/London 23–24th November, 1964 Vol 1 p 2–12

Jellum E, J Aaseth & L Eldjarn Mercaptodextran a metal-chelating and disulphide reducing polythiol of high molecular weight *Biochem Pharmacol* 1973 in press

Longcope W T & J A Luetscher Clinical uses of 2,3 dimercaptopropanol (BAL) XI The treatment of acute mercury poisoning by BAL. *J clin Invest* 1946 25 557–567

Longcope W T Value of BAL in the treatment of poisoning by mercury bichloride *Bull Ayer clin Lab* 1952 4 61–70

Magos L. Effect of 2 3 dimercaptopropanol (BAL) on urinary excretion and brain content of mercury *Brit J Industr Med* 1968 25 152–154

Masui I Experimental Hg poisoning I Effect of sulphur containing compounds and CaEDTA as the antidote Cit from *Chem Abstr* 1957 52 20655d

Nigrović V Der Einfluss von Chelatbildern auf das Verhalten von Quecksilber im Organismus *Arzneimittelforsch* 1963 13 787 790

Randall R. V & A O Seeler Review of medical progress BAL. *New England J Med* 1948 239 1004–1009 1040–1046

Rothstein A & A Hayes The metabolism of mercury in the rat studied by isotope technique *J Pharmacol exp Therap* 1960 130 166–176

Stocken L A & R H S Thompson Reactions of British anti Lewisite and other metals in living systems *Physiol Rev* 1949,

Ulfvarson U Some experiments with antidotes against rats *Proc XIV Congr Occupat Health* Madrid .

Waters L. L & C C. Stock BAL (British Anti lewis

Acta pharmacol et toxicol 1973, 32, 442–448

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Kidney Changes in Mice due to Oriental Hornet (*Vespa orientalis*) Venom: Histological and Electron Microscopical Study

By

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(Received August 17 1972 Accepted November 24 1972)

Abstract Administration to mice of *Vespa orientalis* venom was found to cause acute tubular necrosis of the kidney The proximal tubular epithelial cells showed an abundance of autophagic vacuoles suggesting disintegration of mitochondria A few necrotic cells were found The mechanism of the action of the venom on the kidney tubule cells is as yet unknown but there is evidence for direct effect of the venom on the mitochondria

Key words Mice – *Vespa orientalis* venom – kidney changes – histology

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The venom of the Oriental Hornet causes a severe haemolysis in mice followed by haemoglobinuria (EDERY *et al* 1972) Clinical communications have reported renal damage followin wasp and bee stings (JONAS & SUGAR 1963 RYSTAND 1955, VENTERS *et al* 1961) In the present study the kidneys of mice after the administration of wasp venom were examined The aim of this study was to verify whether renal changes are secondary to haematuria, or whether the venom acts directly on the kidneys

Material and Methods

Into the tail of 6 male Albino mice weighing approximately 20 g 0 25 saline containing 1 mg of wasp venom sac extract (EDERY *et al* 1972) was injected Six other mice were injected simultaneously with venom plus anti serum against *Vespa orientalis* venom according to the method of ISHAY *et al* (1971) All twelve animals were sacrificed by decapitation 45 minutes after the administration of venom The kidneys were taken out and fixed by immersion in chilled phosphate buffered 2 % glutaraldehyde Part of the kidneys were cut into 1 mm small pieces and put into glutaraldehyde The fixation time was 2 hours The other part was fixed in formaldehyde embedded in paraffin and sections were stained with haematoxylin-eosin and P.A.S.

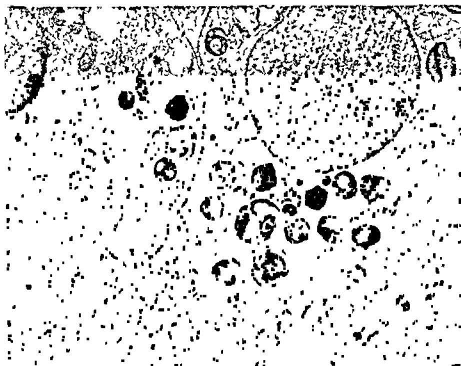


Fig 1 Proximal convoluted tubule, cytoplasm filled with various types of dense bodies Magnification × 4,900

Perl s method (Prussian Blue) for ferric Iron and the Benzidine method for haemoglobin were performed according to PEARCE (1960) The pieces fixed in glutaraldehyde were post fixed in OsO_4 and embedded in Epon 812 Ultrathin sections were stained with lead acetate and uranil nitrate

Results

Clinical observation In 2 mice which received the venom macroscopic haemoglobinuria appeared 30 minutes after venom administration In 4 mice no haemoglobinuria was found The 6 mice which had received the venom gradually became apathetic, they did not move about in their cages and after approximately 30 minutes convulsions occurred. Their breathing became shallow, slow and irregular All of the 6 mice which had received venom and anti-serum simultaneously did not show any clinical or behavioural changes, particularly no haemoglobinuria

Light microscopy Paraffin embedded sections The glomeruli, Henles's tubules, distal tubules, collecting tubules and blood vessels were normal Changes were found in the proximal tubules No casts were found. A special

stain for iron and haemoglobin did not show any positive reaction either in the lumen or in the epithelial lining cells The epithelium of the proximal tubules showed irregular vacuolation of their eosinophilic cytoplasm These vacuoles were of different sizes ranging from 6 7 μ to 1 μ in diameter The vacuoles did not stain with P A S but minute P A S positive pranules were dispersed in their cytoplasm

Electron microscopy The glomeruli appeared to be normal The proximal tubuli showed the following The brush border was normal Only a few apical vacuoles or apical tubular invaginations were seen Within the cytoplasm there were abundant irregular pleomorphic membrane-bound dense bodies of various sizes up to 2–3 μ in diameter (fig 1) These dense bodies were of different configurations Many showed a lamellar structure (fig 2) while others were heteromorphic being partially lamellar and partly composed of compact osmophilic material (fig 3) A few lamellated bodies



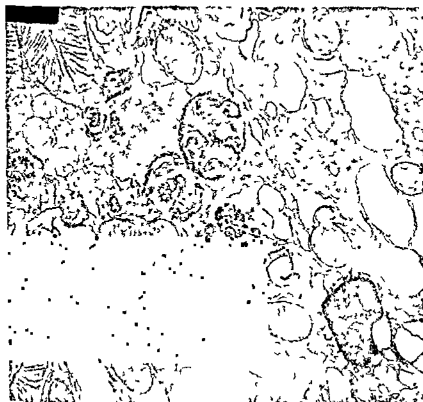


Fig 3 Laminated and dense bodies within cytoplasm. Magnification × 8 000

were encircled by a double membrane, suggestive of a mitochondrial origin (fig 4) A single shrunken necrotic cell was seen in between the epithelial cells (fig 5) No nucleus could be identified in this cell The cytoplasm was composed of irregular vacuoles and an electron dense, unidentified material was noted

The animals which had received venom and anti-venom simultaneously did not show any pathological changes

Discussion

The effect of haemolysis with haemoglobinuria on the proximal tubules epithelium was studied extensively by Ericson (1964) using exogenous haemoglobin He found considerably enlarged apical tubular invaginations which contained small amounts of flocculent or finely granular material Apical vacuoles corresponding to large pinocytotic vacuoles containing concentrated solution of tubular fluid were prominent In the present study, neither apical tubular invagination nor apical vacuoles were enlarged or

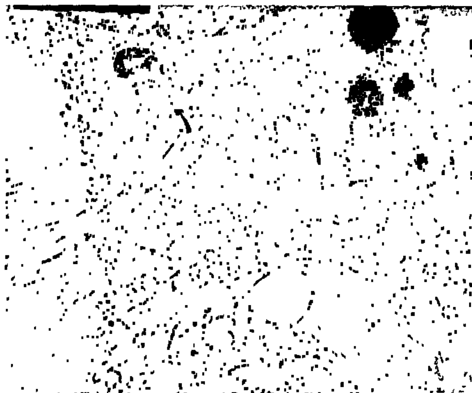


Fig 4 Double membrane surrounding lamellar body (arrow) Magnification × 9,200

more numerous No ferritin-like material could be identified in the tubular lamina or cells Thus it appears that the lesions of the renal tubular epithelium were not related to the haematuria. This is supported by the fact that in 3 animals without haematuria the lesions were identical with those in the animals with haematuria

In the present study, the mitochondrial changes were similar although not identical to those found in Guinea-pig muscle after venom administration (SANDBANK *et al* 1971) Other investigators have also found that Hymenopterae venom affects mitochondrial function (HABERMANN 1968) It has recently been reported that the effect of lipase on functional and morphological mitochondria integrity is manifested by an increased mitochondrial fragility and marked degenerative changes in mitochondrial ultrastructure (FRENCH *et al* 1971, HABERMANN 1972) Since numerous lipolytic enzymes are present in the venom, functional and morphological mitochondrial changes must be expected

As mentioned above, no indication for heterophagy was observed in the proximal tubular epithelium No casts were seen within the lumina and no evidence was observed of excessive function in the apical region of the

epithelial cells The findings seem to represent a process of engulfment and digestion of the tubular cells' own organelles, i e. a process of autophagocytosis. The lamellar appearance with myelin-like figures suggests that at least some of the autophagic bodies are derived from mitochondrial phagocytosis (ARSTILA & TRUMP 1968).

Increased numbers of autophagic vacuoles appear following a variety of injuries to the renal tubule (HRUBAN *et al.* 1963) The presence of necrotic tubular cells in the same tubule with cells loaded with autophagic vacuoles suggests *acute tubular necrosis* (TRUMP *et al* 1969) *The precise mechanism* of tubular necrosis induced by *Vespa orientalis* venom is as yet not resolved Whether the lesions are the result of a direct effect of the venom on the tubular epithelial cells, or whether hypoxia, hypotension or shock are responsible remains to be elucidated As mitochondrial changes were induced both in the tubular epithelium and striated muscle (SANDBANK *et al* 1971), it may be assumed that the venom acts directly on mitochondrial function and ultra-structure.

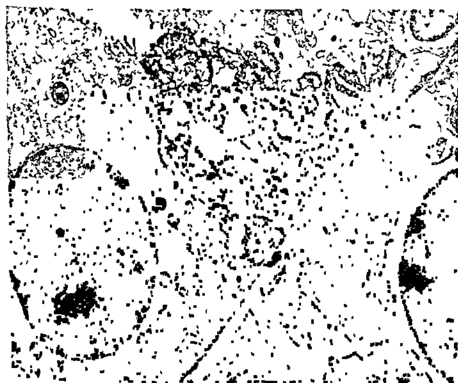


Fig 5 Single necrotic epithelial cell in between apparently normal cells. Magnification × 3,400

REFERENCES

Arstila, A V & B F Trump Studies on cellular autophagocytosis The formation of autophagic vacuoles in the liver after glucagon administration *Amer J Path* 1968, 53, 687–733

Edery, H J Ishay, I Lass & S Gitter Pharmacological activity of Oriental hornet (*Vespa orientalis*) venom *Toxicon* 1972, 10, 13–23

Ericson, J L E Absorption and decomposition of homologous hemoglobin in renal proximal tubular cells *Acta Path Microbiol Scand* 1964 Suppl 168, 1–121

French, S W, M L Norum, T J Ihrig & T Todoroff Effect of phospholipid hydrolysis on the structural and functional integrity of mitochondria *Labor Investig* 1971, 25 427–434

Habermann, E *Rev Physiology, Biochemistry and Exp Pharmacology* Springer Verlag, Berlin 1968, Vol 60 p 220

Habermann, E Bee and wasp venoms *Science* 1972, 144, 314–322

Hruban, Z, B Spargo, H Swift, R W Wissler & R G Kleinfeld Focal cytoplasmic degration *Amer J Path* 1963, 42, 657–683

Ishay, J, S Gitter & J Fischl The production and effectivity of rabbit antiserum against *Vespa orientalis* venom *Acta Allergol* 1971, 26, 286–290

Jonas, W & M Sugar Severe hepatic and renal damage following wasp stings *Dapim Refuiim* (Tel Aviv) 1963, 22, 353–356

Pearse, A G E Histochemistry, theoretical and applied Second Edition, Churchill Ltd, London, 1960

Rystand D R Onset of the nephrotic syndrome during a reaction to bee sting *Stanf Med Bull* 1955, 13 227

Sandbank, U, J Ishay & S Gitter Mitochondrial changes in the Guinea pig muscle after envenomation with *Vespa orientalis* venom *Experientia* 1971, 27, 303–304

Trump, B F, C C Tischer & A J Saladino The nephron in health and disease In *The biological basis of medicine* Ed E E Bittar Academic Press New York 1969, Vol 6, p p 387–494

Venters H D Bee sting nephrosis A study of the immuno pathologic mechanism. *Amer J Dis Child* 1961, 102, 688–689

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Cortisol Distribution in Rheumatoid Arthritis During Treatment with Gold

By

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Abstract Twentyfour patients with rheumatoid arthritis (A R A criteria) were examined before and after a period of gold treatment (10–33 weeks) The Rheumatoid Activity Index (according to LANSBURY 1958) decreased significantly (mean ± S D −33 2 ± 26 P < 0 01) as did the tissue concentration of cortisol (skin biopsies at 8 a m) The values decreased on an average from 51 ng/g to 20 ng/g the mean decrease ± S D being 31 ng/l ± 55 (P < 0 02) However no significant correlation could be demonstrated between the fall in the Activity Index and the decrease in tissue cortisol No consistent changes were found in the plasma cortisol (total or ultra filtrate) Similar to other non steroid anti inflammatory drugs gold appears to exert a "non specific" influence on the distribution of endogenous cortisol

Key words Aurothiosulphate – cortisol – protein binding – rheumatoid arthritis – man

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The possible relation between the action of non steroid anti rheumatic drugs and the distribution of endogenous cortisol has been considered in several investigations Phenylbutazone, indomethacin and probably other drugs seem to affect either the protein binding (BRODIE 1965) or the tissue concentration of cortisol (HVIDBERG *et al* 1971, JANSEN & SCHOU 1971 & 1972) However, a possible connection between these findings and the basic mechanism of action of these drugs has not been established The present work is a continuation of a series of studies from this laboratory about the influence of anti rheumatic drugs on the distribution of cortisol The purpose has been to evaluate any possible changes in plasma and tissue cortisol in patients with rheumatoid arthritis during gold therapy

Methods and Material

Patients Over a period of 19 months (thus covering all seasons of the year) 34 patients were included in the study (21 females and 13 males) All patients satisfied the criteria for the diagnosis of rheumatoid arthritis as given by the *American Rheumatism Association* (1959) However, only 24 patients (14 females and 10 males) completed the study Five patients were excluded because of unsuccessful analyses Five patients experienced severe side effects (exanthema) which, in all cases led to cessation of the gold injections and immediate treatment with prednisone

Investigational design Patients were included in the study only after a couple of weeks without any other medication *Evaluation of the rheumatoid activity* was done before and after treatment with gold according to LANSBURY's Rheumatoid Activity Index (LANSBURY 1958) *Cortisol measurements* On the same day as the Activity Index was measured blood samples and tissue specimens were obtained for the determination of cortisol concentrations The sampling of blood and tissue specimens was always performed at 8 a m according to the procedure described previously (HVIDBERG *et al* 1968) The tissue was obtained by punch biopsy in the gluteal region after local anaesthesia with ethylene dichloride The concentration of cortisol was determined in plasma, ultra filtrate of plasma and in tissues by a micro-spectrophotofluorometric technique (JANSEN *et al* 1967) The ultra filtration method has been described previously (JANSEN & SCHOU 1971)

Therapeutic regimen Sodium aurothiosulphate (natrii aurothiosulfas NFN, sanocrysin®, was given intravenously in increasing doses once a week starting with 25 mg and then in increasing doses to 250 mg The duration of the gold treatment varied between 10 and 32 weeks, depending on the rate at which the doses were increased, which, in turn was governed by the reaction to the treatment The total dose amounted on an average to 2500 mg (1500–3100 mg) No other medication was given during the period of gold treatment except for aspirin

Results

In table 1 the results obtained before the treatment (including also the excluded patients) are compared with previous results (HVIDBERG *et al* 1968 & 1971) No differences were found between the various groups of patients Most of the 24 patients who completed the study improved during the gold treatment, as can be seen in the decrease of the Rheumatoid Activity Index (fig 1)

The post treatment values for the various cortisol determinations are compared with the pre-treatment values and the differences are tabulated in table 1

The reduction in tissue cortisol (table 1, fig 2) is statistically significant, which is not the case for the plasma cortisol (total or ultra-filtrate) An attempt was made to correlate the reduction in tissue cortisol to the rate of improvement according to the Activity Index for all 24 patients, but no correlation could be demonstrated (fig 3) Attempts to correlate the changes

Table 1

The average values and changes in the Activity Index and of the cortisol concentrations in various groups of patients

	Activity Index mean ± S D		Cortisol concentration					
			Plasma (total) mean ng/ml ± S D		Ultrafiltrate mean ng/ml ± S D		Tissue (skin) mean ng/g ± S D	
	initial	Δ	initial	Δ	initial	Δ	initial	Δ
esent material								
oup 1 (n=24) (completed)	94 4 ± 24	−33 2 ± 26 (P < 0 01)*)	123 ± 47	−8 2 ± 55 (n s)	13 ± 7 6	−1.2 ± 9 7 (n s)	51 ± 56	−31 ± 55 (P < 0 02)*)
oup 2 (n=10) (excluded)	79 9 ± 38	–	111 ± 44	–	14 ± 7 0	–	53 ± 82	–
evious material								
ntrols (VIDBERG *et al* 1968)	–	–	122 ± 43	–	–	–	57 ± 34	–
ntrols			112 ± 32		6 7 ± 3 8		46 ± 26	
llagen diseases (VIDBERG *et al* 1971)			136 ± 56		–		78 ± 44	

= the difference between pre treatment and post treatment values in the present investigation
s gnificantly different from zero according to t test

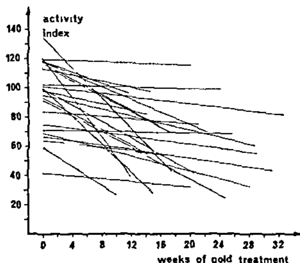


Fig 1 The changes in the Rheumatoid Activity Index (according to LANSBURY) (1958) in 24 patients treated with gold for 10 to 33 weeks

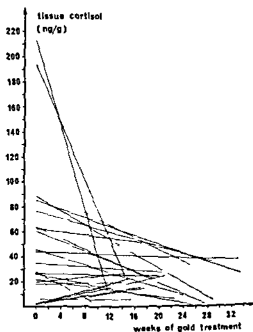


Fig 2 The development of the tissue (skin) concentration of cortisol in 24 patients with rheumatoid arthritis treated with gold for 10 to 33 weeks

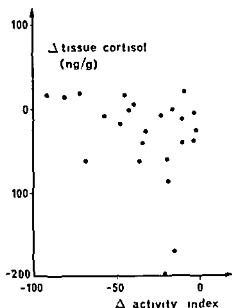


Fig 3 The relation between the pre and post treatment values of the LANSBURY Activity Index (1958) and the tissue cortisol concentrations No significant correlation was observed

in tissue cortisol to the pre-treatment values of the Activity Index, the duration of the treatment, the total dose of gold given or the sex, respectively, were also negative Nor could any correlation be found between any of the alterations in the plasma cortisol and the changes or the pre-treatment values of the Activity Index, respectively The five patients with side effects did not differ significantly from the remaining material with regard to the pre treatment values

Discussion

The object of the present study was solely to investigate the cortisol distribution during gold therapy of rheumatoid arthritis and thus not designed as a controlled therapeutic trial Whether the improvement seen in most of the patients was due to the gold therapy or merely occurred by coincidence cannot be settled, although the former is more likely The average decrease in tissue cortisol was significant following gold therapy for several months and the mean concentration at this time was considerably lower than usually observed in other investigations (HVIDBERG *et* ' ‹ & 1971) However, on the basis of the present investigational des

be decided whether this decrease was a result of the improvement itself, the gold treatment or possibly even of other factors Thus, the altered cortisol distribution may not at all be connected with the actual therapeutic mechanism of action in rheumatoid arthritis Such a relation is even less probable, since no alteration was found in the plasma cortisol and no correlation was demonstrated between the fall in tissue cortisol and the improvement of the Activity Index

Previous results with anti-inflammatory drugs point in the same direction A tendency to lower tissue cortisol was found in guinea pigs treated with phenylbutazone (JANSEN & SCHOU 1971) and the same tendency was demonstrated in patients treated with indomethacin for more than three weeks (HVIDBERG *et al* 1971) However, patients treated for 2–3 years did not show the same picture In rats treated with gold SAWYER *et al* (1964) observed an increased *in vitro* hepatic metabolism of cortisone, but it was not measured whether the distribution of endogenous glucocorticoids had been altered

The results seem to point to a more "unspecific" influence on the cortisol distribution of several anti rheumatic compounds A causal relationship between anti-rheumatic therapy and the peripheral cortisol distribution can, however, not be demonstrated

REFERENCES

A Committee of the American Rheumatism Association Diagnostic criteria for rheumatoid arthritis *Ann Rheum Dis* 1959 18 49–53

Brodie B B Displacement of one drug by another from carrier or receptor sites *Proc Royal Soc Med* 1965 58 946–955

idberg E J Schou & J Aas Jansen Influence of indomethacin on the distribution of cortisol in man *Eur J Clin Pharmacol* 1971 3 102–105

idberg E J Schou J Aas Jansen & J E Clausen Tissue and plasma cortisol in man under various conditions *Acta Med Scand* 1968, 184 215–223

sen J Aas & J Schou The distribution and metabolism of cortisol under the influence of non steroidal antirheumatic drugs *Acta pharmacol et toxicol* 1971, 30 153–157

sen J Aas & J Schou Cortisol distribution and metabolism in guinea pigs after high doses or treatment for three weeks with indomethacin phenylbutazone or chloroquine *Acta pharmacol et toxicol* 1972 31 209–216

sen J Aas, E Hvidberg & J Schou A spectrophotofluorometric micromethod for the determination of cortisol (hydrocortisone) in plasma and tissue obtained by needle biopsies *Scand J Clin Lab Invest* 1967 20 49–56

Lansbury, J Report of a three year study on systemic and articular rheumatoid arthritis *Arthr and Rheum* 1958 1 505–522.

Sawyer, N J, D M Mills & R C Troop Relationships between reticuloendothelial system stimulation adrenal steroid metabolism and the inflammatory response *Curr Therapeut Res* 1964 6, 532–537

Acta pharmacol et toxicol. 1973, 32, 455–466

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Excretion of Some Drugs in Bovine Tears

By

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Abstract The excretion of drugs in the tears of cattle was studied, while almost constant plasma concentrations were maintained by continuous intravenous infusion The examination included both acid and basic drugs A fairly constant ratio between the concentrations in the tears and the ultrafiltrate of plasma was found for each drug examined The ratio was not influenced by the rate of secretion or the plasma concentration For drugs of an acid nature the ratio was less than or equal to 1 (sulphanilamide 0 99, sulphadimidine 0 80, sulphadiazine 0.35, sulphadoxine 0 48, benzylpenicillin 0 004), whereas the ratio for drugs of a basic nature was greater than 1 (penethamate 7 1, trimethoprim 2.2) The above-described investigations provide reasons for believing that the passage of the examined drugs from the plasma to the tears may take place by diffusion of the non protein bound, unionized fraction

Key words Drug excretion – bovine tears

Comprehensive studies by RASMUSSEN (1966) have shown that the transfer of drugs from blood to milk occurs by diffusion of the unionized, non-protein-bound fraction Similar diffusion of drugs across other biological membranes in the organism has been demonstrated, e g the gastric mucosa (SHORE *et al* 1957), the intestinal mucosa (SCHANKER *et al* 1958), the salivary gland (KILLMANN & THAYSEN 1955) and the sweat gland epithelium (THAYSEN & SCHWARTZ 1953) The principles involved in the passage of drugs from blood to tears have apparently not been described although BALÍK (1965) has shown that sulphonamide is excreted in the tears The aim of the present investigation was to elucidate some aspects of the excretion of drugs in bovine tears

Materials and Methods

The excretion of the following drugs was determined sulphanilamide, sulphadimidine, sulphadiazine sulphadoxine, benzylpenicillin, penethamate hydroiodide (Leocillin) and 2,4 diamino-5 (3,4,5 trimeth oxybenzyl) pyrimidine (Trimethoprim)

The animals under study showed no signs of ocular disorders The experiments with sulphanilamide sulphadimidine, and sulphadiazine were performed on 4 calves of the Red Danish Milkbreed The calves weighed from 101–310 kg The excretion of each of the three sulphonamides was determined in 6 experiments In two of the calves (J No 181, 182) two experiments were made with each sulphonamide, one at a low and one at a high plasma level The excretion of sulphadoxine and trimethoprim was determined in 7 experiments using 3 Jersey cows (F3, F4, F5) The excretion of benzyl penicillin and penethamate, respectively, was examined in 3 experiments on 2 calves (J No 182, 184)

The drug solutions were infused into the left jugular vein through a plastic catheter After infusion of an initial dose, constant intravenous infusion was established to maintain a constant plasma concentration (RASMUSSEN 1958) Throughout the experiments with penethamate the solution was cooled to 0° to reduce the rate of hydrolysis Blood samples were drawn from the right jugular vein through a permanent cannula

Tears were sucked from the lower conjunctival sac by means of a 1 000 μl constriction pipette Apart from the mechanical irritation caused by the collection procedure tear secretion was not stimulated in any other way Results of electrolyte analyses of the collected fluid (PEDERSEN, unpublished results) showed a great similarity with the data obtained in man (THAYSEN & THORN 1954) and rabbits (YOSHIMURA & HOSOKAWA 1963) indicating that a representative sample of tears was obtained by the method used by the author Specific stimulation with pilocarpine and tear gas was attempted but higher rates of tear secretion were not obtained Each sample consisted of approximately 2 ml fluid The "flow rate" was tentatively defined as the volume of tears collected per minute In each experiment five tear samples were collected at intervals of 15–30 min A blood sample was drawn immediately before each collection of tears In the experiments with the sulphonamides, penethamate and trimethoprim the first sample was taken 30–45 min after the establishment of constant intravenous infusion In the experiments with benzylpenicillin the initial period was 120 min before the ratio between the concentrations of penicillin in the plasma and tears remained constant

The pH of tears was determined by means of a pH meter connected with a microcapillary glass electrode (Type G 297, Radiometer, Copenhagen) at a temperature of 38° The tear fluid was sucked directly from the conjunctival sac into the thermostated electrode The measurement was performed immediately after each collection of tear sample Determination of pH was moreover carried out under standardized conditions at 38° after equilibration with an O_2–CO_2 mixture at a carbon dioxide pressure of 40 mmHg (JØRGENSEN & ASTRUP 1957, AALUND & NIELSEN 1960) The pH of the blood samples was measured immediately after sampling

The sulphonamide analyses were carried out according to the method of BRATTON & MARSHALL (1939) Determination of urea was performed by the micro-diffusion method (CONWAY 1950) Ultrafiltration of the plasma samples was carried out according to POULSEN'S (1956) method Ultrafiltration of tear samples was not performed partly because of the small volumes available partly because of the low protein content (PEDERSEN & NANSEN 1972) Determination of antibiotics was performed by the agar

cup method using *Sarcina lutea* (ATCC 9341) as a test organism As regards penethamate determination of benzylpenicillin was performed after extraction with butyl acetate as described by HALLAS MØLLER *et al* (1952) Trimethoprim was determined by the spectrofluorimetric method described by SCHWARTZ *et al* (1969)

Assuming a non ionic diffusion through the epithelium the theoretical distribution ratios (R) between the concentrations of non protein bound drug in tears and plasma can be calculated according to the formulae

$$R = \frac{1 + 10^{(pH_T - pK_a)}}{1 + 10^{(pH_B - pK_a)}} \text{ for acids (1)}$$

$$R = \frac{1 + 10^{(pK_a - pH_T)}}{1 + 10^{(pK_a - pH_B)}} \text{ for bases (2)}$$

in which pH_T is the pH of tears (pCO_2 40 mmHg) and pH_B is the pH of blood (vide RASMUSSEN 1966) The following pK_a values were used for the calculations sulphanilamide 10 4, sulphadimidine 7 4, sulphadiazine 6.5 sulphadoxine 6 1, benzylpenicillin 2 7 penethamate 8.5 and trimethoprim 7 6

The results given in the tables are the average of 5 periods in each experiment The analyses were carried out as duplicate determinations

Results

Sulphonamides

The concentrations of the sulphonamides were lower in tears than in plasma The ratios between the concentrations in tears and plasma are shown in table 1 In plasma, part of the sulphonamide was bound to protein The extent of the protein-binding varied for the respective sulphonamides (table 1) After correction for protein-binding in plasma there was a nearly constant ratio between the concentrations of sulphonamide in tears and the ultrafiltrate of plasma for each individual sulphonamide For sulphanilamide an average ratio (T P ultr) of 0 99 was obtained For sulphadimidine, sulphadiazine, and sulphadoxine the average ratio was 0 80, 0.35, and 0 48, respectively The ratios between the concentrations in tears and ultrafiltrate of plasma were unaffected by the sulphonamide concentration in the plasma (fig 1) The ratios were also independent of variations in "flow rate" from less than 0 1 to approximately 0 6 ml per minute

Benzylpenicillin

The results of the excretion experiments are shown in table 2 In one of the experiments, where the plasma concentration was 2 4 i u /ml, only traces of penicillin could be detected in tears In the other two experiments the ratio between the concentrations of penicillin in tears and plasma was 0 002 0 003, respectively After correction for protein binding in plasma between the concentrations of benzylpenicillin in tears and plasma was 0 003 and 0 005 The ratio was independent of "flow rate" from less than 0 1 up to

Concentrations of sulphonamides in tears and plasma and in ultrafiltrates of plasma, and experimentally found and theoretically calculated distributions between tears and ultrafiltrates of plasma

	J No	Plasma μg/ml	Tears μg/ml	Ratio T P	Protein bind plasma %	Ultra filtr plasma μg/ml	Experimental T P ultr	Blood pH	Tears pH	Theoretical T P ultr
Sulphanilamide	181	26.8(26.1– 27.7)	20.6	0.77	19.1	21.7	0.95	7.47	7.27	1.00
	182	28.4(27.4– 30.0)	22.7	0.80	27.1	20.7	1.10	7.48	7.27	1.00
	182	53.7(53.0– 55.2)	40.7	0.76	18.7	43.7	0.93	7.47	7.28	1.00
	184	77.7(76.1– 81.5)	64.0	0.82	21.7	60.8	1.05	7.45	7.30	1.00
	181	88.6(78.8– 95.1)	67.4	0.76	21.3	69.7	0.97	7.48	7.28	1.00
	183	112.5(110.0–114.1)	82.5	0.73	20.2	89.8	0.92	7.50	7.31	1.00
Sulphadimidine	183	74.3(71.7– 76.1)	15.0	0.20	75.6	18.1	0.83	7.47	7.32	0.84
	181	101.5(97.8–106.5)	24.6	0.24	72.7	27.7	0.89	7.51	7.31	0.79
	182	109.5(106.5–113.5)	28.8	0.26	67.2	35.9	0.80	7.49	7.33	0.83
	182	129.9(129.1–131.8)	35.9	0.28	64.3	46.4	0.77	7.48	7.28	0.80
	181	189.1(184.8–195.7)	60.2	0.32	57.4	80.6	0.75	7.47	7.29	0.82
	183	228.3(226.1–232.1)	78.1	0.34	54.1	104.8	0.74	7.51	7.35	0.83
Sulphadiazine	181	70.2(64.3– 73.9)	16.4	0.23	19.8	56.3	0.29	7.52	7.26	0.59
	182	71.6(69.6– 75.0)	18.0	0.25	30.1	50.0	0.36	7.45	7.27	0.69
	182	82.3(81.5– 82.9)	21.9	0.27	23.0	63.4	0.35	7.48	7.39	0.83
	184	108.7(103.3–114.1)	33.5	0.31	24.5	82.1	0.41	7.50	7.27	0.63
	183	132.6(129.9–138.6)	33.4	0.25	24.1	100.6	0.33	7.54	7.33	0.65
	181	193.5(190.2–198.4)	50.2	0.26	23.1	148.8	0.34	7.45	7.27	0.69
Sulphadoxine	F5	43.2(38.9– 47.5)	7.4	0.17	73.5	11.4	0.65	7.43	7.29	0.74
	F4	56.3(51.4– 64.6)	6.0	0.11	77.3	12.8	0.47	7.50	7.28	0.62
	F3	217.7(215.9–221.3)	42.5	0.20	50.4	108.1	0.39	7.43	7.29	0.74
	F5	232.0(219.5–244.4)	61.7	0.27	43.6	130.8	0.47	7.46	7.32	0.74
	F5	248.0(240.9–273.0)	58.2	0.23	46.9	131.7	0.44	7.47	7.30	0.69
	F4	253.7(246.2–264.0)	58.4	0.23	49.2	128.8	0.45	7.46	7.30	0.70
	F4	263.0(244.4–278.3)	59.1	0.22	50.7	129.7	0.46	7.47	7.32	0.72

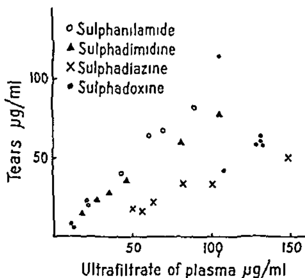


Fig 1 Relation between the concentrations of sulphanilamide sulphadimidine sulphadiazine and sulphadoxine in tears and ultrafiltrate of plasma

Penethamate

Penethamate hydroiodide is an ester which in itself is antibiotically inactive, its hydrolytic degradation product, benzylpenicillin, being the active principle Penethamate is a base The concentrations of penicillin determined as benzylpenicillin, in tears and plasma are shown in table 3 It appears that the concentration in tears was found to be about twice as high as the concentration in plasma samples taken at the same time Ultrafiltration was not performed Fractional determination of ester and free penicillin was carried out in two experiments The results of these experiments are shown in table 4 In one of the experiments where the benzylpenicillin concentration in the plasma was 0 36 i u /ml, penicillin could not be demonstrated in tears In the other experiment, where the average plasma concentration was 0 88 i u /ml, only traces were detectable in tears The concentration of penethamate was 5 6 and 8 6 times higher in tears than in plasma The ratio (T P) was independent of variations in ' flow rate ' from 0 1–0 5 ml per minute

Trimethoprim

The ratio between the concentrations of trimethoprim in tears and plasma varied from 0 6–1 2 (table 5) After correcting for protein binding in plasma the average ratio between the concentrations in tears and ultrafiltrate of plasma was 2 2 The ratio was unaffected by the concentration in plasma and independent of variation in "flow rate from 0 1–0 6 ml per minute

Table 2

Concentrations of benzylpenicillin in tears and plasma and in ultrafiltrates of plasma and experimentally found and theoretically calculated distributions between tears and ultrafiltrates of plasma

J No	Plasma i u /ml	Tears i u /ml	Ratio T P	Protein bind Plasma %	Ultra filtr Plasma i u /ml	Experimental T P ultr	Blood pH	Tears pH	Theoretical T P ultr
184	2 4(2 1–2 6)	Trace	–	26	1 8	–	7 47	7 25	0 60
182	6 9(6 7–7 3)	0 011	0 002	36	4 4	0 003	7 47	7 27	0 63
184	8 3(7 7–8 9)	0 028	0 003	30	5 8	0 005	7 48	7 28	0 63

Table 3.

Total concentrations of penicillin, determined as benzylpenicillin, in plasma and tears after intravenous infusion of penethamate

J No	Plasma i u/ml	Tears i u/ml	Ratio T P
182	0 75(0 64–0 95)	1 4	1 9
182	0 76(0 65–0 87)	1 6	2 1
184	1 6 (1 4 –1 8)	3 2	2.0

Urea

The concentrations of urea were determined in tears and corresponding plasma samples The average ratio between the concentrations in tears and plasma water was 0 53±0 004 (n=174) (mean±S E M) It was independent of the plasma concentration (fig 2) and of variations in "flow rate" from less than 0 1 to approximately 0 6 ml per minute The high plasma values were obtained by oral administration of urea Urea is a base with a pK_a value of 0 2

The pH of tears

In the present study the pH of tears was measured by sucking the fluid directly from the conjunctival sac into a capillary glass electrode, thus providing anaerobic conditions The values observed varied from 7 30–7 58, mean 7 44±0 007 (n=92) As a general rule the highest values were found when the "flow rate" was low, and the lowest values were found when the rate of secretion was high Since the pH of tears varies with the secretion rate or with the time in which the fluid is exposed to the air, the pH of all

Table 4

Concentrations of penethamate and benzylpenicillin in tears and plasma after intravenous infusion of penethamate and experimentally found and theoretically calculated distributions of penethamate between tears and plasma

J No	Penethamate Plasma i u/ml	Penethamate Tears i u/ml	Penethamate Experimental T P	Penethamate Blood pH	Penethamate Tears pH	Penethamate Theoretical T P	Benzyl penicillin Plasma i u/ml	Benzyl penicillin Tears i u/ml
182	0 25	1 4	5 6	7 45	7 28	1 4	0 36	0
184	0 44	3 8	8 6	7 46	7 30	1 4	0 88	Trace

Table 5

Concentrations of trimethoprim in tears and plasma and in ultrafiltrates of plasma and experimentally found and theoretically calculated distributions between tears and ultrafiltrates of plasma

J No	Plasma µg/ml	Tears µg/ml	Ratio T P	Protein bind Plasma %	Ultra filtr Plasma µg/ml	Experimental T P ultr	Blood pH	Tears pH	Theoretical T P ultr
F5	1 0(0 9–1 1)	0 6	0 6	66	0 3	2 0	7 46	7.30	1 3
F3	1 2(0 9 1 2)	1 1	0 9	51	0 6	1 8	7 48	7 26	1 4
F3	1 6(1 3–1 8)	1 8	1 1	53	0 8	2 3	7 48	7 28	1 3
F4	1 8(1 7 2 1)	2 2	1 2	57	0 8	2 8	7 46	7.30	1 3
F4	2 0(1 8 2 3)	2 1	1 1	58	0 8	2 6	7 58	7 31	1 4
F5	3 7(3 2–4 2)	3.3	0 9	57	1 6	2 1	7 39	7 31	1 1
F5	7 8(7.5–8 6)	6 8	0 9	53	3 7	1 8	7 46	7.32	1 2

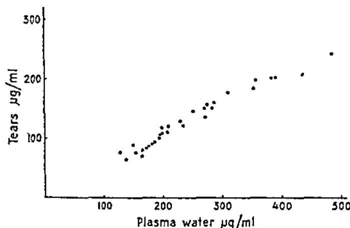


Fig. 2. Relation between the concentration of urea in tears and plasma water

the samples was also measured under standardized conditions, i.e. after equilibration with an oxygen-carbon dioxide atmosphere of a carbon dioxide pressure of 40 mmHg and at a temperature of 38°. Determined in this way the pH of tears was 7.29 ± 0.003 ($n = 157$). The pH of the blood varied from 7.39–7.58 (tables 1, 2, 4 and 5).

Discussion

The results show that the ratios between the concentrations of the respective drugs in tears and in ultrafiltrate of simultaneously drawn plasma samples were fairly constant. The ratio was not influenced by the plasma concentration or by the rate of secretion. For drugs of an acid nature the ratio was less than or equal to 1, whereas for drugs of a basic nature the ratio was greater than 1. These findings may suggest that the excretion of the drugs examined in tears occurs by diffusion of the unionized, non protein-bound fraction.

By comparing the ratios found in the experiments with the theoretically calculated distribution ratios (the T/P ultr. ratio in table 1) good agreement was found in sulphanilamide and sulphadimidine. Owing to its high pK_a value (10.4) sulphanilamide is present only in the unionized form both in the plasma and tears. For the more highly ionized compounds sulphadiazine and sulphadoxine, the experimentally found ratios were lower than the theoretical ones.

For benzylpenicillin there was poor agreement between the experimental findings and the calculated theoretical distribution of the drug between tears

and ultrafiltrate of plasma (the T P ultr ratio in table 2) The theoretically expected ratio is approximately 0 6 which contrasts markedly with a ratio of only 0 003 and 0 005 obtained in the experiments The reason for this discrepancy is not known A partial explanation could be the very low content of unionized penicillin in the plasma (less than 0 001% at pH 7 6), a low diffusibility of penicillin (RASMUSSEN 1966) and probably as yet unknown factors

For the bases penethamate and trimethoprim the ratios found experimentally were higher than the calculated theoretical values (table 4 and 5) As mentioned above the reverse was true for the partly ionized drugs of an acid nature This could mean that the pH measured in tears is not the true pH of the fluid as it is secreted by the lachrymal gland

Determination of pH in tears involves a number of problems In man the pH values of tears vary greatly Some investigators have observed a neutral *reaction (pH about 7 0), or more often a slightly alkaline value, about that* of serum (7 3–7 5), while others have found a still more alkaline reaction (cf NORN 1968) The reason for the varying results is that tears contain a considerable amount of carbon dioxide which escapes when the tear fluid is exposed to the air, resulting in increasing pH By dripping an indicator solution directly into the conjunctival sac, thus, avoiding any great loss of carbon dioxide, NORN (1968) found that the reaction of the tear fluid most often lies about the neutral point (pH 7 0) or on the acid side of this (pH 6 8) In order to compensate for this loss of free carbon dioxide in the present investigation the pH of tears was also measured after the samples had been brought into equilibrium by means of a controlled atmosphere of a carbon dioxide pressure about that of arterial blood, i e 40 mmHg (DONAWICK & BAUE 1968) Measured by this method, the pH of tears (7 29±0 003) was always lower than the pH of blood

The present investigation revealed that the concentration of urea in tears was about half that measured simultaneously in the plasma Since all urea in plasma is present in unionized form, one might have expected that the concentrations would be similar in plasma and tears, as they are in the case of sulphanilamide This result is not in agreement with the findings of THAYSEN & THORN (1954) in man and BRUSILOW (1967) in cats who found identical urea concentrations in tears and in plasma Likewise in cows and goats, RASMUSSEN (1961 & 1966) found constant identical concentrations of urea in samples of blood and milk collected simultaneously In pancreatic juice from two dogs the secretion to plasma concentration ratio for urea was found to be 0 8 and 0 9, respectively (BRO-RASMUSSEN *et al* 1956)

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REFERENCES

Aslund, O & K Nielsen Determination of standard bicarbonate in blood. Investigations on the acid base metabolism in cattle and swine *Nord Vet Med* 1960, **12**, 605–620

Balik, J Methodische Bemerkung zur biochemischen Tranenuntersuchung *Acta Opthal* 1965, **43**, 190–198

Bratton, A C & E K Marshall A new coupling component for sulfanilamide determination *J biol Chem* 1939, **128**, 537–550

Bro-Rasmussen, F, S A Killman & J H Thaysen The composition of pancreatic juice compared to sweat, parotid saliva and tears *Acta physiol scand* 1956, **37**, 97–113

Brusilow, S W Evidence for a non plasma source of urea in sweat *Nature* 1967, **214** p 506

Conway, E J *Microdiffusion analysis and volumetric error* Crosby Lockwood & Son Ltd, London, 1950

Donawick, W J & A E Baue Blood gases, acid base balance, and alveolar arterial oxygen gradient in calves *Amer J vet Res* 1968 **29** 561–567

Hallas Møller, K, H O Juncher, C Møller & B Wille Nogle kemiske og biologiske undersøgelser over et organspecifikt penicillinderivat Hydroiodidet af benzylpenicillin diethylaminoethanolester (Leocillin) *Ugeskr Læg* 1952 **114** 447–456

Jørgensen K & P Astrup Standard bicarbonate its clinical significance and a new method for its determination *Scand J clin Lab Invest* 1957 **9** 122–132

Killmann, S A & J H Thaysen The permeability of the human parotid gland to a series of sulfonamide compounds paraamino-hippurate and inulin *Scand J clin Lab Invest* 1955 **7**, 86–91

Norn, M S Hydrogen ion concentration of tear fluid Estimated on the basis of a bromthymol blue dyed lacrimal streak *Acta Ophthal* 1968, **46** 189–200

Pedersen, K B & P Nansen Immunoglobulins in bovine lachrymal fluid *Acta path microbiol scand Sect B* 1972 **80**, 231–240

Poulsen, E Renale clearanceundersøgelser hos køer Thesis Copenhagen 1956

Rasmussen, F Mammary excretion of sulphonamides *Acta pharmacol et toxicol* 1958, **15** 139–148

Rasmussen, F Mammary excretion of antipyrine, ethanol and urea *Acta vet scand* 1961, **2** 151–156

Rasmussen F Studies on the mammary excretion and absorption of drugs Thesis Copenhagen 1966

Schanker, L S, D J Tocco, B B Brodie & C A M Hogben Absorption of drugs from the rat small intestine *J Pharmacol exp Therap* 1958, **123** 81–88

Schwartz, D E, B A Koechlin & R E Weinfeld Spectrofluorimetric method for determination of trimethoprim in body fluids *Chemotherapy* 1969,

Shore, P A, B B Brodie & C. A M Hogben The gastric secretion of drugs A pH partition hypothesis *J Pharmacol exp Therap* 1957, **119**, 361–369

Thaysen, J H & I L Schwartz The permeability of human sweat glands to a series of sulphonamide compounds *J exp Med* 1953, 98, 261–268

Thaysen, J H & N A Thorn Excretion of urea, sodium, potassium and chloride in human tears *Amer J Physiol* 1954 **178**, 160–164

Yoshimura, H & K Hosokawa Studies on the mechanism of salt and water secretion from the lacrimal gland *Jap J Physiol* 1963, 13, 303–318

Acta pharmacol et toxicol 1973, 32, 467–480

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The Functional Role of Cholinergic Receptors in the *Outlet Region of the Urinary Bladder:* An *in Vitro* Study in the Cat

By

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Abstract A method for experimental study *in vitro* of the outlet region (bladder base bladder neck and proximal urethra) from the cat is described which allows simultaneous recording of resistance to flow through the region and of isometric tension in the longitudinal muscles The results suggest that acetylcholine (ACh) may influence the smooth muscles in the outlet region by several mechanisms Contractions in the longitudinal muscles are mediated by atropine sensitive cholinergic receptors The resistance to flow could be either increased or decreased following ACh stimulation and in neither case could the response be blocked with atropine If the response was an increase however, it was blocked by phentolamine and if it was a decrease, it was blocked by propranolol It is concluded that ACh stimulates the circular musculature in the outflow region through α and β adrenergic receptors It is suggested that the effects of ACh on flow are brought about through short intramural adrenergic neurons ACh stimulates their ganglion-cells which results in a release of nor adrenaline at the nerve endings and in activation of adrenergic receptors in the circular muscle layer

Key words Bladder – neurogenic bladder – autonomic nervous system – sympathetic nervous system

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The localization and function of autonomic receptors in the bladder and proximal urethra in man and cat was recently described (NERGÅRDH & BOREUS 1972) It was found that cholinergic receptors as well as β-adrenergic receptors were generally distributed in the dome of the bladder, in the bladder base and in the proximal urethra, whereas α-adrenergic receptors could be demonstrated only in the base of the bladder and in the proximal urethra In the case of the cholinergic and the α adrenergic receptors, pharmacological stimulation resulted in contraction of the smooth muscles, while stimulation

of the β adrenergic receptor produced relaxation No species differences between man and cat were found

These findings prompted us to a further analysis of the receptor pharmacology of the outlet region, i e the bladder base, bladder neck and proximal urethra The presence of a closing mechanism in this region has been accepted by most authors It has been ascribed to either a passive mechanism depending on the elasticity of the tissue (LAPIDES 1958, WOODBURNE 1961, RUCH 1965) or to an active muscle contraction initiated by the sympathetic nervous system (ELLIOTT 1907, LEARMONT 1931, EDVARDSEN 1967) Our results on the localization and function of the α adrenergic receptor in isolated muscle strips are in agreement with the "active" theory If a passive closing mechanism exists the outflow region must be assumed to be opened by contraction of longitudinal muscle bundles in the bladder neck when micturition takes place This seems to be supported by our previous finding (NERGÅRDH & BORÉUS 1972) that the parasympathetic transmittor acetylcholine (ACh) produces contraction in muscle strips from the outflow region taken parallel with the longitudinal axis of the bladder However, if the strips are taken perpendicular to the longitudinal axis the same type of contraction is obtained following stimulation with ACh Such a contraction could certainly not play a part in an opening mechanism

A further analysis made an experimental technique necessary which allowed functional studies of the outflow region under more physiological conditions To meet these requirements a method was developed which is described in the present report It is used to characterize the cholinergic receptor function in the bladder base, bladder neck and proximal urethra

Material and methods

Thirty five adult cats 24 males and 11 females weighing 2.5 kg were used The animals were anaesthetized with pentobarbital (mebumalum NFN) 30–50 mg/kg intraperitoneally An abdominal incision was made and the bladder neck and proximal urethra were dissected without dislodging the bladder which was usually filled with urine Two small sutures were made into the wall of the urethra and the distance between them was measured This distance was maintained during the entire experiment in order to ensure the same tension of the isolated preparation as that of the preparation *in situ* The urethra as well as the ureters were ligated and cut and the whole bladder dissected free It was placed in Tyrode solution (NaCl 8 g KCl 0.2 g $CaCl_2$ 0.2 g $MgCl_2$ $6H_2O$ 0.1 g $NaHCO_3$ 1 g NaH_2PO_4 H_2O 0.05 g glucose 1 g distilled water ad 1000 ml) of room temperature Care was taken to keep the tissue moist during the preparation

The upper part of the bladder was cut away by means of a transverse section immediately above the entrance of the ureters in the bladder The content of urine in

the bladder was usually about 30 ml, but the individual variation was considerable The preparation then consisted of the bladder base, bladder neck and proximal urethra A thin steel ring (10 mm ⌀) was sutured to the proximal end of the preparation in order to maintain the *in situ* anatomy of the bladder base and neck (In some experiments anterograde perfusion was used and the steel ring was then replaced by a catheter with the same diameter It was fixed to the bladder wall with a ligature at the same place as the ring) A catheter (diameter 2 42 mm) was introduced from the distal end of the urethra in such a way that the distance between the end of the catheter and the plane of the ureters was either 1 or 2 cm after applying the final tension to the preparation The catheter was fixed by a ligature

The preparation was mounted in a 20 ml overflow type bath (fig 1) with Tyrode solution at 37° The pH was maintained at 7 5 by bubbling a mixture of 93 5 vol % O_2 and 6 5 vol % CO_2 through the solution The catheter in the urethra was attached to a cannula through the bottom of the bath and the steel ring was connected to a strain gauge transducer so that the isometric tension of the longitudinal musculature of the urethra could be recorded on a Polygraph

The preparation could be perfused through the cannula and the urethral catheter with Tyrode solution (37°, pH 7 5) from a reservoir A constant flow, usually 10–12 ml/min, was maintained by means of a Harvard tubing pump Changes in resistance to flow were recorded with a Statham Pressure Transducer Usually the direction of

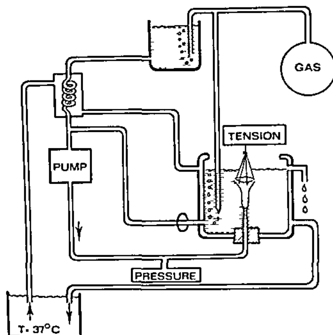


Fig 1 Arrangement for constant flow perfusion of the outlet region of the bladder The resistance to flow was recorded as pressure of the perfusion fluid The isometric tension in the longitudinal direction was simultaneously recorded with a strain gauge transducer The bath fluid could be exchanged intermittently by means of a stop-cock

flow was retrograde but several experiments were also made with anterograde perfusion in order to check that the same results were obtained irrespective of direction of flow After mounting in the bath, the preparation was allowed to stabilize during at least one hour before the experiment started

Drugs Acetylcholine iodide (ACh) atropine sulphate, phentolamine (regitin®) and propranolol (inderal®) were added either directly to the bath in volumes of 0 1–0 3 ml or to the perfusion fluid in the reservoir

Results

With the technique described above it was possible to measure the effects of cholinergic stimulation both in terms of isometric tension in the longitudinal smooth muscles and in terms of resistance to flow The possible influence on the resistance to flow of various experimental factors like the tension of the preparation, the angle between the bladder base and the urethra, and the rate of flow was first investigated (see below) The influence of the antagonists atropine, phentolamine and propranolol on the effect of ACh-stimulation was then analyzed individually and in combination on a qualitative basis (five preparations) Atropine was used to block cholinergic receptors, phentolamine and propranolol were given in order to explore whether the effect of acetylcholine on the resistance to flow involved adrenergic receptors A survey of the results from 35 cat experiments is given in table 1

Basal conditions The initial resistance to flow following mounting of the preparation in the bath as described above and after a resting period of one hour was 4–10 cm H_2O The rate of flow could be varied between 5 and 40 ml Tyrode solution/min without measurable change in basal resistance The drug induced changes in resistance which will be described below are expressed as either an increase or decrease from this initial level of resistance

The basal resistance decreased continuously during the experiment with about 0 5 cm H_2O per hour It was influenced by the longitudinal tension of the preparation If the tension was decreased the resistance increased and vice versa The isometric tension of the logitudinal smooth muscle was very stable during the whole experiment

Cholinergic receptors Effects on the longitudinal smooth muscles

Stimulation with ACh invariably resulted in a contraction which was dose related but independent of whether the agonist was injected into the bath fluid or mixed in the perfusion fluid The strength of the response increased if the preparation had been stretched before addition of ACh However, initial over stretching to more than 50 % of the length *in situ*

Table 1

Experimental findings (35 cats)

Sex	Basal resist. cm H_2O	ACh effect on resistance to flow (A) in % of basal resistance and on isometric tension in grams (B) 0 5 µg/ml A	0 5 µg/ml B	1 5 µg/ml A	1 5 µg/ml B	5 µg/ml A	5 µg/ml B	15 µg/ml A	15 µg/ml B	50 µg/ml A	50 µg/ml B	500 µg/ml A	500 µg/ml B	Comments	Conclusions regarding flow resistance
Female	10	0	0 1			+4	0 4			+20	1 4				Increase
Female	5	0	0 3			+50	1 6			*+100*	3 0			Fig 2 I	Increase
Female	10									+60	4 4			ACh in perfusion fluid	Increase
Female	4			−7	0 8			−10	4	−14	6 8				Decrease
Female	4			−12	1 4	−25	3	−50	4 4						Decrease
Female	2	0	0 4			−25	1 6	−50	3 8						Decrease
Female	6	0	0 1			0	0 4	0	0 6						No reaction
Female	5	0	0 3			−10	1 4			−35	4 4			Fig 2 II	Decrease
Female	3	0	0 1			−13	0 8			−13	3 6			Blockade Propr Phent. Atrop	Diphasic
										+50					
Female	7	0	0 1			+3	0 7			+50	3 8			Blockade Propr	Increase
Female	8									+10	2			Blockade Phent. Propr Atrop	Increase
Male	4	0	0 04			+5	0 16			+30	0 76				Increase
Male	13	0	0 3			−3	0 8			−20	1 6				Decrease
Male	5					0	0 08			+6	0 2	+12	0 7		No reaction
Male	10	0	0 5			+23	1 8			+66	5 0				Increase
Male	5	0	1 7			−8	6	−12	7 5						Decrease
Male	10	0	0 2			+2	1 6			+17	5 1				Increase

Male	13	0	0 8		−10	2 9			−21	6 2				Decrease
Male	5				+25	0 4			+125	1 4				Increase
Male	5								±66	3 3			ACh in perfusion fluid	Diphasic
Male	10	0	0 1		0	0 6			+75	2 7			ACh in perfusion fluid	Increase
Male	5	0	0		+4	0 2			+10	0 8				No reaction
Male	5	0	0 1		+8	1 3			+83	5 1				Increase
Male	5	0	0 2		0	1								No reaction
Male	5	0	0 05		+7	0 4	+7	1						No reaction
Male	4						+19	1 7	+55	2 8				Increase
Male	2	0	0		0	0 2			0	1				No reaction
Male	6	0	0 1		−5	0 6			−15	1 8				Decrease
Male	10	0	0 1		+10	1			+60	6				Increase
Male	3	0	0 1		+25	0 5			+150	1 7				Increase
Male	6	0	0		+4	0 2			+17	0 5				Increase
Male	7	0	0 2		+8	1 8			+25	4 8				Increase
Male	3	0	0		0	0 4			+33	2 6			Blockade Atrop Phent	Increase
Male	5	0	0 1		+10	1			+12	4			Blockade Phent.	Increase
Male	7				+5	0 2					+130	2 4		Increase

resulted in a decrease of the response The rate of flow could be varied between 5 and 40 ml/min without influencing the isometric response of the longitudinal muscle

The threshold ACh concentration for contraction was usually 0 5 µg/ml The contraction was very stable and no significant fatigue was observed when the preparation was perfused with the agonist

Cholinergic receptors Effects on resistance to flow

At low concentrations of ACh it was possible to demonstrate contraction of the longitudinal muscle as described above without any change in the

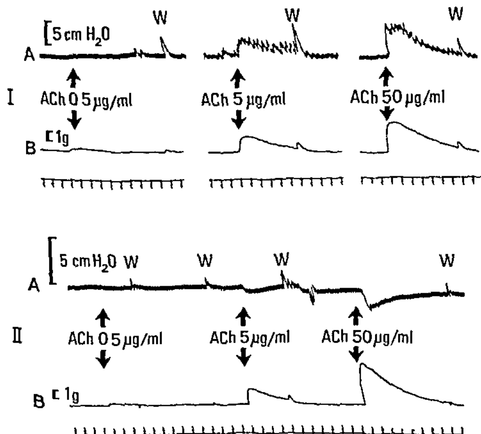


Fig 2 Perfusion experiments in two cats (I and II) A Resistance to flow B Isometric tension Note that in cat I the resistance to flow always increased when acetylcholine (ACh) was added to the bath In cat II the response was always a decrease in resistance The isometric tension however always increased when ACh was given. All were dose-dependent W = washout of drugs Time scale in minutes

resistance to flow At somewhat higher concentrations of ACh, given either directly to the bath or via the perfusion fluid, the resistance to flow increased in 19 preparations, decreased in 8 and was diphasic or nil in 8 The increase as well as the decrease was dose dependent (fig 2) A decrease type of response was more common in specimens taken from female than from male cats

This was a seemingly confusing pattern with regard to the opinion that the outflow region is opened via parasympathetic impulses (see below) The influence of stretching on resistance to flow during ACh perfusion was therefore investigated in preparations that had initially responded with an increase in the basal resistance This could be done by adjusting the length of the specimens with a screw device It was found that a slight stretching of the preparation resulted in lowering of the ACh response It was, however, impossible to change an initial increase in the basal resistance into a decrease by stretching the tissue On the other hand, if the length of the preparation was decreased, ACh stimulation resulted in an increase in resistance to flow No change in response to ACh was observed when the angle between the bladder base and urethra was varied by changing the distance between the steel ring and the urethra or by tilting the plane of the ring in relation to the urethral axis Variation in the rate of flow from 5 to 10 ml/min did not change the response to ACh (50 μg/ml perfusion fluid) (At higher rates of perfusion the response to ACh stimulation was potentiated)

Cholinergic receptors Effects of atropine

Perfusion of the preparation for 30 minutes with 0 1 μg/ml atropine in the perfusion fluid resulted in a complete blockade of the isometric response of the longitudinal muscle to ACh However, no simultaneous blockade of the ACh induced effects on resistance to flow was observed The atropine inhibition was slowly reversible

Cholinergic receptors Effects of phentolamine

Perfusion was made with 0 5 μg phentolamine/ml Tyrode solution Preliminary experiments had shown that this concentration produced a complete blockade of the alpha adrenergic response of the preparation Addition of ACh to the bath produced the same or a slightly reduced contraction of the longitudinal muscle as that obtained before phentolamine exposure The effect of ACh on resistance to flow, however, was modified by phentolamine, in all cases a decrease from the basal resistance was noted This means that preparations which had previously responded to ACh with an increase now reacted with a decrease (fig 3) The response to ACh during a blockade was dose-dependent The blockade of phentolamine was reversible

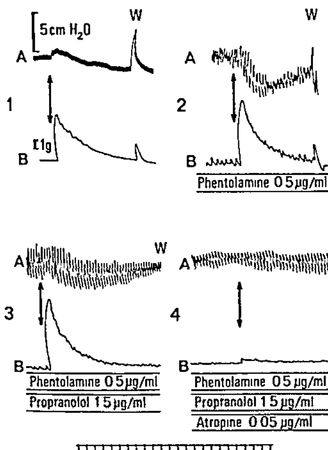


Fig 3 Perfusion of the outlet region from one cat when responses to acetylcholine (ACh) were modified by successively added antagonists Arrows indicate addition to the bath of ACh (150 µg/ml)

1 Increased flow resistance (A) and increased isometric tension in longitudinal direction (B) when ACh was given with no inhibitor present
2 During perfusion with phentolamine The response was now changed to a decrease in resistance Note the increase in spontaneous activity
3 During perfusion with phentolamine and propranolol The decrease in resistance was now almost completely blocked
4 During perfusion with phentolamine, propranolol and atropine All responses to ACh were now abolished The effects of the antagonists were reversible

Cholinergic receptors Effects of propranolol

Preliminary experiments with propranolol had shown that a concentration of 0 5–5 µg/ml perfusion fluid produced a 50–100 % blockade of the β-adrenergic receptor function Following 30 minutes perfusion, ACh was

added directly to the bath This resulted in a slightly increased contraction respone of the longitudinal muscle as compared to the pre blockade situation Likewise, the basal resistance to flow increased if ACh was added to the bath during propranolol perfusion If the preparation had responded to ACh by a decrease before blockade, this response was changed to an increase in the presence of propranolol The inhibition by propranolol was reversible

Cholinergic receptors Effects of combination of atropine, phentolamine and propranolol

A combined blockade was produced by means of addition to the perfusion fluid of the three antagonists in the same concentrations as described above (fig 3) A sufficient time period was allowed to ensure the blockade of the antagonists after which ACh was added directly to the bath The results were compared to those before addition of the inhibitors

The response of the longitudinal smooth muscle was completely abolished when all three inhibitors were present As stated above, atropine alone is responsible for this blockade

The initial ACh effect on resistance to flow, irrespective of whether it was an increase or a decrease, was abolished if phentolamine and propranolol were present simultaneously in the perfusion fluid

Discussion

The experimental approach presented in this paper proved to work very well and the receptor functions in the outlet region could be successfully analyzed avoiding the complicating factor of concomitant changes in the intravesical pressure The results suggest that ACh can influence the smooth muscles in the bladder base, bladder neck and proximal urethra by several mechanisms Contractions in the longitudinal muscles of the outflow tract was mediated by atropine sensitive cholinergic receptors α and β-adrenergic blockade did not interfere with this mechanism

A more complicated situation was found with regard to the resistance to flow which probably mainly reflects the tension in the circular muscle This resistance could either increase or decrease on ACh stimulation and in neither case was the response significantly influenced by atropine α adrenergic blockade, however, changed an ACh induced increase in resistance to flow into a decrease, and this decrease could then be blocked by a β adrenergic antagonist. On the other hand, if the initial ACh response had been a decrease in resistance this was changed to an increase in resistance following ACh if a β-blocker was present If both α- and β-adrenergic receptors were blocked, ACh was always without effect on the resistance to flow

Our interpretation of these findings is that ACh stimulates the longitudinal musculature mainly through cholinergic receptors, and the circular musculature mainly through α- and β-adrenergic receptors

Our hypothesis is also borne out by the scattered data in the literature ELLIOTT (1907) found that stimulation of the pelvic nerve in the cat decreased the resistance to flow in the urethra except in two cases where instead a marked increase in resistance was noted

It was shown by LEARMONT (1931) in man that the internal sphincter is influenced by inhibitory fibres through the parasympathetic nervous system and also through excitatory fibres in the sympathetic. On the other hand, he pointed out that the internal sphincter remained closed following sympathectomy, but that it was relaxed when the parasympathetic system was also injured His conclusion was that parasympathetic activity in some unknown way, may close off the outflow region

TANAGHO *et al* (1969) showed that the smooth muscle in the [illegible]

[illegible]

sponse was reported to be sluggish and gradual The authors concluded that this reaction had no physiological significance KLEEMAN (1970) demonstrated that the choline ester bethanechol decreased the resistance to flow in the dog urethra Finally, HOMSY (1967), also working on dogs, found that stimulation of the pelvic nerve in most cases did not influence the resistance to flow during perfusion of the bladder neck but that, in a few cases a decrease in resistance occurred

All these data demonstrate that ACh, released from nerve endings or administered to the experimental system may either increase, decrease or have no effect at all on the resistance to flow through the outlet region [illegible]

[illegible]

muscle layer with the remainder of the detrusor and its [illegible] of this innervation has been looked upon as a mechanism [illegible] opening in which the increase in the intravesical pressure also plays a part [illegible] *et al* [illegible]

[illegible]

stimulation produced a contraction of the longitudinal muscle [illegible] in so far as [illegible] region. However, only in some experiments a decrease was found [illegible] tance to flow In fact, an increase in resistance was more common

be concluded that the shortening of the outflow region due to the contraction of the longitudinal muscle is not essential for the functional opening of the bladder ELLIOTT (1907) arrived at a similar conclusion based on his demonstration that a decrease in resistance to flow by means of stimulation of the pelvic nerve was not associated with a simultaneous shortening of the urethra

Our experiments suggest that ACh may operate through different receptor mechanisms in the bladder musculature The ACh induced contraction of the longitudinal muscle was easily antagonized by atropine, whereas the action of ACh on resistance to flow was difficult to block with this antagonist It has been suggested in the literature, on the basis of comparison between exogenously administered acetylcholine and endogenously released transmitter, that different cholinergic receptors have a different "sensitivity" to atropine (URSILLO 1961, CARPENTER 1963, CHESTER 1970) The present investigation demonstrates that the "atropine sensitive" effect is produced at the cholinergic muscle receptor whereas the "atropine resistant" reactions are mediated through adrenergic α- and β receptors in the muscle We suggest that the ACh effect on flow is brought about through stimulation of the intramural sympathetic ganglia *This is a new concept and its relations to* previously reported data in the literature as well as its consequences will be discussed below

The outflow region has been extensively studied during recent years with histochemical methods and the results are quite compatible with our proposal that ACh may interfere with the adrenergic neurons producing muscle effects through adrenergic receptors in the effector organ The early studies by KUNTZ & MOSELEY (1936) have been followed by histological investigations of the intramural innervation of the bladder and urethra in the cat by HAMBERGER & NORBERG (1965), EL BADAWI & SCHENK (1966, 1968) and OWMAN *et al* (1971) All these investigators have been able to demonstrate intramural adrenergic ganglion cells belonging to the sympathetic nervous system These cells are especially abundant in the muscle of the bladder base (EL BADAWI & SCHENK 1966) and in the proximal urethra (OWMAN *et al* 1971) The latter authors also showed that hypogastric denervation caused a decrease in the number of adrenergic nerve fibres in the muscle wall concomitant with a significant decrease in the amount of noradrenaline in the tissue On the basis of such denervation studies they concluded that about ⅔ of the adrenergic nerves in the muscle layer consisted of short sympathetic neurons with intramural ganglion cells Both HAMBERGER & NORBERG (1965) and EL BADAWI & SCHENK (1966) emphasize adrenergic connections with cholinergic ganglia The latter authors also state that fibres exist which connect intramural parasympathetic with sympathetic ganglia The histochemical findings are consistent with our interpretation of

the action of ACh on resistance to flow According to this view, ACh stimulates the short sympathetic neurons via their intramural ganglion cells The result is a release of noradrenaline at the nerve endings which activates the adrenergic receptors in the muscle cell Since these short adrenergic neurons are concentrated to the circular muscle layer of the proximal urethra (OWMAN *et al* 1971) the ACh effect on the ganglia will mainly be an influence on the resistance to flow These effects are consequently "atropine-resistant", but may be blocked by specific adrenergic antagonists, e g propranolol or phentolamine

Definite evidence for this hypothesis would only be possible in our system by means of a ganglionic blockade of exclusively sympathetic intramural ganglia Since there is no drug available at present that is selective enough to achieve this effect, such an approach has not been possible However, experiments on ganglionic blockade (SIGG & SIGG 1964, EDVARDSEN 1968, LA GRANGE 1971) have suggested that endogenously released ACh in some way interferes with adrenergic nerve endings

An alternative explanation for our experimental results would be offered by the 'cholinergic link hypothesis" (BURN & RAND 1959) FERRY (1966) in discussing this theory suggested that ACh may stimulate calcium uptake at adrenergic nerve terminals, thereby causing release of noradrenaline Experimental evidence in favour of the hypothesis is, however, considered to be incomplete (Ferry 1966, Koelle 1972)

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REFERENCES

Burn J H & M J Rand Sympathetic postganglionic mechanism *Nature* 1959 184 163–165

Carpenter F G Excitation of rat urinary bladder by coaxial electrodes and by chemical agents *Amer J Physiol* 1963, 204 727–731

Chester G B Differentiation of receptors for exogenous and endogenous acetylcholine in the urinary bladder *Agents and Actions* 1970 1 128–132

Edvardsen P Nervous control of urinary bladder in cats *Acta Neurol Scand* 1967, 43 543–563

Edvardsen P Nervous control of urinary bladder in cats III Effects of autonomic blocking agents in the intact animal *Acta physiol scand* 1968 72 183–193

El Badawi, A & E A Schenk Dual innervation of the mammalian urinary bladder A histochemical study of the distribution of cholinergic and adrenergic nerves *Amer J Anat* 1966, 119, 405–428

El Badawi, A & E A Schenk A new theory of the innervation of bladder musculature Part I Morphology of the intrinsic vesical innervation apparatus *J Urol* 1968, 99, 585–587

Elliott, T R The innervation of the bladder and urethra *J Physiol* 1907, 35, 367–445

Ferry, C B Cholinergic link hypothesis in adrenergic neuroeffector transmission *Physiol Rev* 1966, 46, 420–456

Hamberger, B & K A Norberg Adrenergic synaptic terminals and nerve cells in bladder ganglia of the cat *Int J Neuropharmacol* 1965 4, 41–45

Homsy, G E The dynamics of the ureterovesical and vesicourethral junctions II Effect of autonomic and somatic excitation on the resistance to flow of the distal ureter and lower urinary tract *Invest Urol* 1967, 4, 408–418

Kleeman, F J The physiology of the internal urinary sphincter *J Urol* 1970, 104, 549–554

Koelle, G B Acetylcholine – current status in physiology, pharmacology and medicine *New Engl J Med* 1972, 286, 1086–1092

Kuntz, A & R L Moseley An experimental analysis of the pelvic autonomic ganglia in the cat *J Comp Neurol* 1936, 64 63–75

La Grange, R G Peripheral autonomic regulation of the canine urinary bladder *Invest Urol* 1971, 9, 64–81

Lapides J Structure and function of the internal vesical sphincter *J Urol* 1958, 80, 341–353

Lapides, J, E P Ajemian, B H Stewart, B A Breakey & J R Lichtwardt Further observations on the kinetics of the urethrovesical sphincter J Urol 1960, 84, 86–94

Learmont, J R Contribution to neurophysiology of the urinary bladder in man *Brain* 1931, 54, 147–176

Nergårdh, A & L O Boreus Autonomic receptor function in the lower urinary tract of man and cat *Scand J Urol Nephrol* 1972 6, 32–36

Owman, Ch., T Owman & N O Sjoberg Short adrenergic neurons innervating the female urethra of the cat *Experientia* 1971, 27, 313–315

Ruch, T C The urinary bladder In *Physiology and biophysics* Ed T C Ruch and H D Patton W Saunders Company, Philadelphia and London 1965, 1010–1021

Sigg E B & T D Sigg *Sympathetic stimulation and blockade of the urinary bladder in cat* *Int J Neuropharmacol* 1964, 3, 241–251

Tanagho, E A, F H Meyers & D R Smith Urethral resistance Its components and implications I Smooth muscle component *Invest Urol* 1969, 7, 136–149

Ursillo, R C Investigation of certain aspects of atropine resistant nerve effects *J Pharmacol Exp Therap* 1961, 131, 231–236

Woodburne, R T *The sphincter mechanism of the urinary bladder and the urethra* *Anat Rec* 1961, 141, 11–20

Acta pharmacol et toxicol 1973, 32, 481–486

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Effects of Membrane Stabilizers on Glucuronidation and Amino Acid Transport in Cultures of Rat Hepatoma Cells

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Abstract Promazine and imipramine in concentrations between 0 005 mM and 0.20 mM and thioridazine between 0 005 mM and 0 10 mM inhibit p aminophenol glucuronidation in rat hepatoma cell cultures The cardiac stabilizers propranolol and quinidine have little or no effect on glucuronidation In a homogenate system prepared from the cells promazine thioridazine and imipramine between 0 1 and 1 0 mM also inhibit p aminophenol glucuronidation In addition promazine thioridazine and imipramine inhibit alanine uptake/incorporation α aminoisobutyric acid uptake and efflux in the cultures of hepatoma cells

Key words Membrane stabilizers – glucuronyl transferase – amino acid transport

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The term membrane stabilizers is being used to designate chemical substances which at low concentrations protect erythrocytes and cell organelles against lysis Promazine (LANDMARK & ØYE 1971), thioridazine (SEEMAN & WEINSTEIN 1966) imipramine (LANGSLET *et al* 1971) and propranolol (LANGSLET 1970) all inhibit hypotonic haemolysis These drugs also reduce cardiac contractile force, decrease cardiac contraction rates and potassium efflux in the isolated rat heart and are probably secondary effects due to stabilization of cardiac cell membranes (LANDMARK 1971a, LANGSLET *et al* 1971, LANGSLET 1970)

A possible membrane stabilization of the liver cell membrane could lead to decreased uptake of endogenous and exogenous substances as well as decreased egress of metabolites Earlier studies have shown that both chlorpromazine (DYBING 1972a) and SKF 525-A (DYBING & RUGSTAD 1973) inhibit the glucuronidation of p aminophenol and p nitrophenol, the uptake/incorporation of alanine and the uptake and efflux of α-aminoisobutyric (DYBING 1972b) in a system of rat hepatoma cells grown in culture

likely through actions on the cell membrane The present investigation was carried out to test whether other membrane stabilizers show inhibitory actions similar to those of chlorpromazine and SKF 525-A on glucuronidation and amino acid transport in the hepatoma cell cultures

Material and Methods

Methods of cell culture

The clonal strain MH_1C_1 of rat hepatoma cells was grown in Dulbecco s modified Eagle s medium supplemented with 15 % horse serum and 2 5 % foetal calf serum and antibiotics as described by DYBING & RUGSTAD 1972

Cell culture experiments

Replicate subcultures were incubated with *p* aminophenol (PAP) 0 20 mM in fresh serum containing medium without or with the drugs to be tested in a total volume of 10 ml for 4 hours at 37° PAP glucuronide was assayed in aliquots of the incubation mediums according to DYBING & RUGSTAD 1972

Homogenate experiments

The rat hepatoma cells in culture were removed with an ice cold 0 02 % EDTA barbital/NaCl buffer pH 7 4 spun down at 700×g and homogenized for 10 minutes in a glass homogenizer with a teflon pestle in a 125 mM tris maleate buffer pH 7 4 and a pestle speed of 1000 r p m The homogenate was centrifuged at 700 × g for 5 minutes at 4° and the supernatant was used as the source of the enzyme The incubation mixture contained 0 1 ml of homogenate final concentration PAP 0 20 mM, UDPGA 4 0 mM tris maleate 75 mM pH 7 4 $MgCl_2$ 10 0 mM and the drug to be tested at various concentrations in a total volume of 0 5 ml Incubations were carried out in a water shaking bath at 37° for 30 minutes Reactions were stopped with 0 5 ml ice-cold 0.5 M TCA

Alanine uptake/incorporation The amount of radioactivity in cellular proteins and total intracellular content was measured after incubation with ^{14}C alanine 0 5 μci (0 005 mM) for 2 hours at 37° as previously described (DYBING 1972b)

α Aminoisobutyric acid uptake and efflux Cellular uptake after incubating with ^{14}C-AIB 0 5 μci (0 01 mM) for 2 hours at 37° was measured as previously described (DYBING 1972b) In the efflux experiments the appearance of radioactivity in fresh medium without or with promazine thioridazine or imipramine after 60 minutes was estimated after preincubating the cells with 0 01 mM ^{14}C-AIB in medium without any additions for 2 hours at 37°

In all the experiments protein was measured according to the method of LOWRY *et al* (1951) using bovine albumin as standard

Chemicals

Drugs and chemicals were obtained from the following companies *p* aminophenol and quinidine from Norsk Medisinaldepot promazine from Norfarma, thioridazine from Sandoz, imipramine from Dumex desipramine from Geigy, propranolol from ICI, and UDPGA (tri ammonium salt) from Sigma ^{14}C-1 alanine (Amersham specific activity 10 mci/mmol) and ^{14}C 3 α aminoisobutyric acid (New England Nuclear, specific activity 5.3 mci/mmol) were purchased through Norsk Atominstitutt.

Table 1

Effect of various drugs on glucuronidation of PAP in cultures of MH_1C_1 cells PAP 0.20 mM in a serum-containing medium was incubated for 4 hours at 37° without or with drugs in concentrations from 0 005 mM to 0 20 mM Values are means of inhibition in per cent from two flasks for each concentration compared to three controls for each of the experiments.

Drug tested	Per cent inhibition with addition of					
	0 005 mM	0 01 mM	0 05 mM	0 10 mM	0 15 mM	0 20 mM
Promazine	8.5	1 8	13 5	22.5	50 7	65 2
Thioridazine	4 7	10 5	25 0	48 5	*	*
Imipramine	11 3	8 0	26.2	33 0	47 0	63 6
Desipramine	0	0	0	19 2	44 8	70 2
Propranolol	0	5 0	6 0	14 9	13 6	14.2
Quinidine	–	0	–	0	5 7	5.3

* cellular detachment

Results

Table 1 shows the effect of various drugs on the glucuronidation of *p*-aminophenol (PAP) in MH_1C_1 cell cultures The phenothiazines promazine and thioridazine both show a dose dependent inhibition of this process Also the tricyclic antidepressant imipramine as well as its demethylated derivative desipramine share this effect In contrast, propranolol and quinidine have little or no inhibitory actions in the concentration range studied Promazine over 0 20 mM, thioridazine over 0 10 mM, and imipramine over 0 20 mM cause cellular detachment Promazine and imipramine are seen to be ap-

Table 2

Effect of promazine thioridazine, and imipramine on glucuronidation of PAP in homogenates from MH_1C_1 cells Values are means of inhibition from three estimations for each of the concentrations in per cent of means of three controls

Drug tested	Per cent inhibition with addition of					
	0 01 mM	0 05 mM	0 10 mM	0 25 mM	0 50 mM	1 00 mM
Promazine	0	1 8	7 0	10 6	24 9	46 0
Thioridazine	0	0	19 7	34 2	67 4	92 7
Imipramine	0 7	0 7	10 9	17 0	23 1	

Table 3

Effect of promazine 0.15 mM, thioridazine 0.075 mM and imipramine 0.15 mM on amino acid transport and incorporation in MH_1C_1 cells. ^{14}C-ALA 0.005 mM was incubated with replicate subcultures in the incorporation experiment and the experiment measuring total intracellular radioactivity for 2 hours at 37°. ^{14}C-AIB 0.01 mM was incubated with replicate subcultures for 2 hours at 37° in the AIB uptake experiment and for 2 hours preloading in the efflux experiment after which the cells were incubated for 1 hour together with the drugs. Values are means ± S.D. from each 4 parallel flasks; numbers in brackets refer to inhibition in per cent of controls

Mechanism studied	Control CPM/mg prot	With addition of		
		Promazine 0.15 mM CPM/mg prot	Thioridazine 0.075 mM CPM/mg prot	Imipramine 0.15 mM CPM/mg prot
ALA into protein	1595.1 ± 198.2	132.1 ± 10.4 (91.6 %)	82.1 ± 11.2 (94.9 %)	172.0 ± 12.7 (89.0 %)
Total ALA into cells	7356.2 ± 469.7	1868.5 ± 274.8 (74.6 %)	1185.8 ± 144.2 (83.9 %)	2113.9 ± 133.2 (71.3 %)
AIB uptake	3143.7 ± 274.4	2555.3 ± 354.2 (18.8 %)	2125.8 ± 385.4 (32.4 %)	2555.8 ± 385.4 (18.8 %)
AIB efflux	947.9 ± 39.6	586.5 ± 55.3 (38.1 %)	616.0 ± 50.2 (35.0 %)	553.6 ± 84.4 (41.6 %)

proximately equipotent with 50 % inhibition at about 0.15 mM, whereas thioridazine reduces the glucuronidation velocity to 50 % of the controls at about 0.10 mM.

Promazine, thioridazine, and imipramine also inhibit the glucuronidation of PAP in a dose dependent manner in a homogenate system prepared from the cells, but at higher relative concentrations than those giving comparable inhibition in the cell cultures (table 2). Fifty per cent inhibition was obtained with promazine and thioridazine at 1.0 mM, and with thioridazine at 0.30 respectively. Experiments with homogenates were not performed with the addition of serum proteins as in the cell culture experiments, hence protein binding of the drugs must also be considered when comparing the values of table 1 with those of table 2.

Table 3 gives the values of various parameters of amino acid transport and incorporation in the MH_1C_1 cells without or in the presence of promazine 0.15 mM, thioridazine 0.075 mM, and imipramine 0.15 mM. The incorporation of alanine into cellular proteins is almost completely blocked by the three drugs. Most of this inhibition is due to a reduction in the total intracellular accumulation of the labelled amino acid, which indicates that

the transport of alanine is inhibited The uptake of the non metabolizable amino acid AIB is affected much less by the drugs than is alanine uptake/incorporation The decreased net uptake of AIB was shown not to be a result of an increased efflux, in fact promazine, thioridazine, and imipramine also inhibited this process

Discussion

Three psychotropic substances, the aliphatic phenothiazine promazine, the piperidine phenothiazine, and the tricyclic anti depressant imipramine all show inhibitory effects on glucuronidation and amino acid transport in a culture system consisting of living cells derived from a rat hepatoma. These effects are very similar to those seen using chlorpromazine and SKF 525-A (DYBING 1972a DYBING & RUGSTAD 1972, DYBING 1972b)

Qualitatively the actions of promazine, thioridazine and imipramine show similar patterns, but thioridazine seems to be the more potent drug with regard to the parameters tested However, promazine is more potent than thioridazine in experiments with isolated rat atria (LANDMARK 1971a)

Promazine and thioridazine produced a decrease in the rate of contractions and work index in isolated rat atria (LANDMARK 1971a) Imipramine decreased impulse generation and impulse conduction velocity, cardiac contractile force, as well as potassium loss in the isolated rat heart (LANGSLET *et al* 1971) Promazine and thioridazine also reduce the potassium loss from isolated perfused rat hearts (LANDMARK 1971b) and rat atria (LANDMARK *et al* 1972) It is thought that promazine thioridazine and imipramine act as membrane stabilizers and thus cause a depression of cardiac activity

Propranolol over 5×10^{-6} M causes inhibition of hypotonic hemolysis, and decreases excitability contractility and potassium efflux in the isolated rat heart (LANGSLET 1970) These effects are similar to those of chlorpromazine In the rat hepatoma cell culture system however, propranolol has much less effect on glucuronidation than the phenothiazines or imipramine Quinidine also failed to inhibit glucuronidation to any appreciable extent in this system

The MH_1C_1 cell line does not oxidize drugs (DYBING 1972a) Competition for UDP GT by hydroxylated metabolites of promazine thioridazine, or imipramine with PAP should therefore not be expected to occur

It is concluded that promazine thioridazine and imipramine probably exert their effect described in the present paper through interaction with the rat hepatoma cell membranes It is suggested that the term membrane sta lization can be extended to include the effects of drugs in this ... to decreased transport of substances

Acknowledgements

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REFERENCES

Dybing, E Chlorpromazine inhibition of p aminophenol glucuronidation by rat hepatoma cells in culture *Acta pharmacol et toxicol* 1972a 31, 287–295

Dybing, E The inhibitory effects of chlorpromazine and diethylaminoethyl diphenyl valerate (SKF 525 A) on alanine incorporation into protein and α aminoisobutyric acid uptake in rat hepatoma cells in culture *Biochem Pharmacol* 1972b, in press

Dybing, E & H E Rugstad *Para* aminophenol metabolism in an established cell line with liver like functions *Acta pharmacol et toxicol* 1972, 31, 153–160

Dybing, E & H E Rugstad The inhibitory effect of diethylaminoethyl diphenyl valerate (SKF 525 A) on glucuronidation by cultures of rat hepatoma cells *Acta pharmacol et toxicol* 1973, 32, 112–118

Landmark, K. The action of promazine and thioridazine in isolated rat atria 1 Effects on automaticity, mechanical performance, refractoriness and excitability *Eur J Pharmacol* 1971a, 16, 1–7

Landmark, K The effect of thioridazine and promazine in reducing the potassium loss from isolated perfused rat hearts *Acta pharmacol et toxicol* 1971b 29, 49–59

Landmark, K., J F W Haffner & J Setekleiv Reduction in 42K efflux from rat atria by promazine and thioridazine *Acta pharmacol et toxicol* 1972, 31, 54–64

Landmark, K & I Øye The action of thioridazine and promazine on biological membranes Relationship between ATPase inhibition and membrane stabilization. *Acta pharmacol et toxicol* 1971, 29, 1–8

Langslet, A Membrane stabilization and cardiac effects of d,l propranolol, d propranolol and chlorpromazine *Eur J Pharmacol* 1970, 13, 6–14

Langslet, A, W Grini Johansen, M Ryg, T Skomedal & I Øye Effects of dibenzepine and imipramine on the isolated rat heart *Eur J Pharmacol* 1971, 14, 333–339

Lowry, O H, N J Rosebrough, A L. Farr & R J Randall Protein measurement with the Folin phenol reagent *J Biol Chem* 1951, 193, 265–275

Seeman, P & J Weinstein Erythrocyte membrane stabilization by tranquilizers and antihistamines *Biochem Pharmacol* 1966, 15, 1737–1752

Acta pharmacol et toxicol 1973, 32, 487–499

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Determination of Guanethidine in Sympathetic Ganglia

By

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Abstract Guanethidine sulphate was administered intraperitoneally in a dose of 10–60 mg/kg for up to two months to adult male rats and the content of guanethidine in the superior cervical ganglia was determined fluorometrically after combination with ninhydrin in alkaline solution Guanethidine was shown to concentrate in the superior cervical ganglia Twenty four hours after the discontinuation of guanethidine sulphate 20 mg/kg for 14 days the mean total ganglionic content of guanethidine base was 50 ng/ganglion (S D 8 8 n=10) corresponding to 83 µg/g dry weight or 17 µg/g wet weight Following a single injection of guanethidine sulphate and following discontinuation of prolonged administration of the drug the ganglionic guanethidine declined with a half life of 35 hours Most of the ganglia were excised 24 hours after discontinuation of administration of the drug at which time the decline in ganglionic guanethidine was exponential Desmethylimipramine largely prevented the accumulation of guanethidine in the ganglia indicating a mainly intraneuronal localization of guanethidine a localization which was confirmed by microautoradiography Reserpine only lowered the content to a small extent possibly indicating a mainly extragranular localization of guanethidine in the nerve cells The selective morphological effects of guanethidine on sympathetic ganglia previously demonstrated were almost completely prevented by desmethylimipramine but only to a small extent by reserpine SKF 525 A potentiated the ganglionic effects of guanethidine It is assumed that the ganglionic effects are due to a cytotoxic effect of guanethidine concentrating selectively in the sympathetic ganglion nerve cells with the major part being located outside the noradrenaline storage granules as opposed to a mainly intragranular localization in the peripheral sympathetic terminals

Key words Guanethidine – ganglia autonomic

Prolonged intraperitoneal administration of large doses of guanethidine sulphate to the rat induces pronounced biochemical and morphological changes in the superior cervical ganglia (JENSEN HOLM & JUUL 1970a, b

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1971, Burnstock *et al* 1971, Juul & McIsaac 1973) The primary action of guanethidine was assumed to be a damaging effect on the ganglionic nerve cells The chromatolysis caused a partial loss of cholinesterases and noradrenaline from the ganglion and was accompanied by an infiltration of small cells

The purpose of the present investigation has been to study the kinetics of guanethidine in the rat superior cervical ganglion Most of the determina tions were carried out 24 hours after discontinuation to avoid the initial rapid changes in the concentrations of guanethidine A possible relationship between the content of guanethidine in the ganglia and the morphological changes was investigated

Material and Methods

Male Wistar rats weighing 225–400 g at the termination of the experiments (mean weight 285 g) were used Guanethidine sulphate (ismelin®, CIBA) dissolved in saline (10 mg/ml) was administered intraperitoneally once daily at doses from 10-60 mg/kg for 1–63 days. In some experiments the animals were simultaneously treated with guanethidine sulphate and desmethylimipramine chloride (pertofran® GEIGY) 15–30 mg/kg or reserpine (serpasil® CIBA) 0 6–1 0 mg/kg In a single experiment the rats received guanethidine sulphate 10 or 20 mg/kg (two rats at each dose) in combina tion with diethylaminoethyldiphenylpropylvalerate HCl (SKF 525 A) 75 mg/kg for 14 days Following varying periods of discontinuation the animals were killed by ex sanguination under ether anaesthesia the superior cervical ganglia were excised freeze dried and stored at 80° until use

Guanethidine determination Eight pooled superior cervical ganglia from 4 rats were weighed and homogenized in cold perchloric acid using a glass homogenizer Follow ing solvent extraction guanethidine was determined fluorometrically after combina tion with ninhydrin in alkaline solution (Schanker & Morrison 1965) excitation at 402 nm emission read at 500 nm (uncorrected instrument values Aminco-Bowman spectrophotofluorometer) The emission spectra were routinely recorded Readings of the concentrations were performed from a standard curve after correction for tissue "blank" (untreated controls)

The tissue blank" corresponded to less than 20 ng guanethidine per sample The relationship between the amounts of guanethidine and fluorescence was linear be tween 40 and 800 ng/sample The sensitivity of the method was 40 ng guanethidine/ sample corresponding to 5 ng guanethidine per ganglion (twice the tissue blank" with a characteristic spectrum) The recovery following the addition of guanethidine to a ganglionic homogenate was 104 % The reproducibility of the analysis was 7 % (coef ficient of variation) Guanidine creatine and creatinine were not determined with the present solvent extraction procedure Similarly the more polar metabolites of guan ethidine were not shown to be co determined under the present conditions (Schanker & Morrison 1965) Desmethylimipramine reserpine and SKF 525 A did not interfere with the analysis

The doses of guanethidine administered are expressed as the sulphate (ismelin®) whereas the amounts determined are expressed as the free base The molecular weight of guanethidine sulphate is 295 and of guanethidine base 198

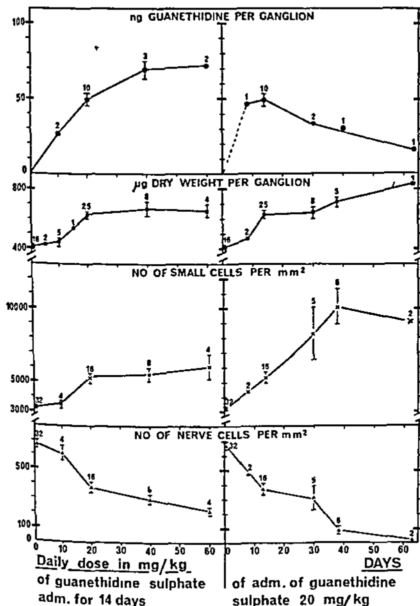


Fig 1 The content of guanethidine base in rat superior cervical ganglia following intraperitoneal administration for 14 days of increasing doses of guanethidine sulphate (*left* part of the figure) and following 20 mg/kg daily for increasing periods of time (*right* part of the figure) The ganglia were excised 24 hours after the last injection The dry weight of the superior cervical ganglia and the number of small cells and ganglion nerve cells per mm² in histological specimens following identical administration of guanethidine sulphate are also shown Each experiment concerning guanethidine content and dry weight of the ganglia is based on analysis of 8 pooled fro-- 4 rats Mean values, S E M, and number of experiments are s

In a single experiment superior cervical ganglia from 5 cats pretreated with guanethidine sulphate 5–40 mg/kg intraperitoneally for 14 days were analyzed

Micro autoradiography One rat weighing 175 g received guanethidine sulphate 20 mg/kg for 11 days followed by a single injection of 12 mg ^{3}H guanethidine sulphate (80 µci per mg) The animal was killed 24 hours later The left superior cervical ganglion was used for micro-autoradiography (BAY, unpublished results), the radioactivity of the right superior cervical ganglion and of the whole animal (mean radioactivity) was measured in a liquid scintillation counter (BROEN CHRISTENSEN 1965)

Histology Histological specimens were stained by the gallocyanine chromalun method of EINARSON (1932) and the silver impregnation method of BODIAN (1936) Cell counts were performed as described by JENSEN HOLM & JUUL (1970b) the cells within an area of 0 1 mm² being counted in each specimen The reproducibility of the cell counts was 2–5 % (coefficient of variation)

The statistical calculations are based on Student s t test or Wilcoxon s test

Results

I Dose time/ganglionic concentrations of guanethidine

The content of guanethidine in *rat* superior cervical ganglia following guanethidine sulphate administered intraperitoneally at various dose levels and for varying lengths of time is shown in fig 1 All the ganglia were excised 24 hours after the last injection After guanethidine sulphate 20 mg/kg for 14 days the ganglionic content was 50 ng per ganglion (S D 8 8, n = 10)

Following 40 mg/kg for 30 and 63 days lower values of guanethidine compared with the 14 day values were observed – similar to the decrease following 20 mg/kg for the same periods of time (fig 1) The values follow-

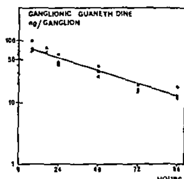


Fig 2 The decrease in the guanethidine base in the rat superior cervical ganglia Values following a single intraperitoneal injection of guanethidine sulphate 60 mg/kg (open circles) and following the administration of 20 mg/kg for 14 days (filled circles) are shown The results obtained 0 5–4 hours after the injection have been omitted Each point represents the analysis of 8 pooled ganglia from 4 rats The line has been drawn according to the method of least squares

ing 40 mg/kg for 14, 30, and 63 days were 57, 39, and 29 ng/ganglion Following 10 mg/kg no decrease with time was observed, the values being 25, 30 and 31 ng/ganglion

The content of guanethidine in the *cat* superior cervical ganglia following the intraperitoneal administration of guanethidine sulphate for 14 days at 5 10, 20 and 40 mg/kg was 25, 94, 96, and 142 ng/ganglion respectively The dry weight of the ganglia was 2 9–4 3 mg

II Ganglionic half life of guanethidine

The decrease of guanethidine in rat superior cervical ganglia following discontinuation is shown in fig 2 Guanethidine sulphate was either administered as a single injection of 60 mg/kg (open circles) or as daily injections of 20 mg/kg for 14 days (filled circles) The dose of guanethidine sulphate of 60 mg/kg used in the acute experiments was calculated according to the results obtained with guanethidine sulphate 20 mg/kg for 14 days in order to attain values of ganglionic guanethidine within the same range Maximal concentrations were obtained 1–4 hours after the injection, but owing to the rapid changes in the concentrations during this period the values were omitted from the figure When the content was plotted against time in a semilogarithmic system a straight line was obtained The line was drawn according to the method of least squares (no deviation from linearity, $P > 0\ 05$) The half life was approximately 35 hours No differences were observed between the results obtained after a single dose and those following prolonged administration

III Effects of desmethylimipramine (DMI) and reserpine on the ganglionic content of guanethidine

The results are shown in fig 3 *DMI* partially prevented the ganglionic uptake of guanethidine both in acute and chronic experiments Following administration for 14 days the DMI insensitive part of the ganglionic guanethidine amounted to 26–33% (9–23 ng per ganglion) of the total ganglionic guanethidine (fig 3 B–E) When the results obtained with various doses of guanethidine sulphate were cumulated the difference was significant ($P < 0\ 01$ Wilcoxon s test)

Although simultaneous administration of *reserpine* did not lower the ganglionic content of guanethidine when the figures were cumulated (fig 3 C–E), lower values were observed in the individual experiments when compared with the corresponding animals treated with guanethidine only, the range of the decrease being 7–14 ng per ganglion This difference was not significant Reserpine only slightly diminished the uptake of guanethidine in acute experiments (fig 3 G), and did not release the guanethidine already taken up by the ganglia (fig 3 F)

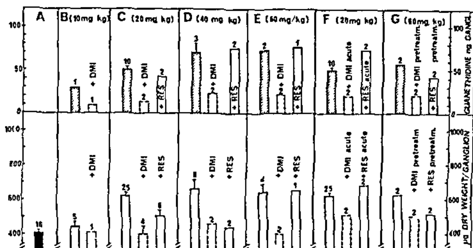


Fig 3 Content of guanethidine base (upper part of the figure) and dry weight (lower part of the figure) of rat superior cervical ganglia following pretreatment of the animals with various drugs A ganglia from untreated control rats B–F pretreatment for 14 days with guanethidine sulphate intraperitoneally at daily doses as stated G single intraperitoneal injection of guanethidine sulphate in the dose stated In B–E the effects are shown of simultaneously administered desmethylimipramine chloride (DMI) 30 mg/kg divided into two daily doses or reserpine (RES) 0 6 mg/kg once daily In F the effect of two single injections of DMI 15 mg/kg and reserpine 0 6 mg/kg administered 24 and 16 hours before killing the animals are shown In G the animals were pretreated with DMI or reserpine in doses as shown above 24 and 16 hours before a single injection of guanethidine sulphate All ganglia were excised 24 hours after the last injection of guanethidine sulphate except for G where the ganglia were excised after 8 hours Each experiments represents the analysis of 8 pooled ganglia from 4 rats Mean values S E M and number of experiments are shown

IV Microautoradiography

Microautoradiography was performed on the superior cervical ganglion from one rat pretreated with guanethidine sulphate 20 mg/kg for 11 days followed by a single dose of ^{3}H guanethidine sulphate 70 mg/kg (a total of 1 2 mci) The animal was killed 24 hours after the administration of labelled guanethidine The result is shown in fig 4 The radioactivity was mainly localized to the ganglion cells The guanethidine content of the contralateral ganglion calculated as guanethidine base was estimated at 27 µg/g wet weight, the whole body content was 10 µg/g

V Effects of guanethidine on ganglionic weight and histology

The dry weight of the superior cervical ganglion was approximately 20% of the wet weight (in control ganglia 23% following pretreatment with guanethidine sulphate 20 mg/kg for 14 days 20% (10 pooled ganglia in each group were weighed before and after freeze-drying))

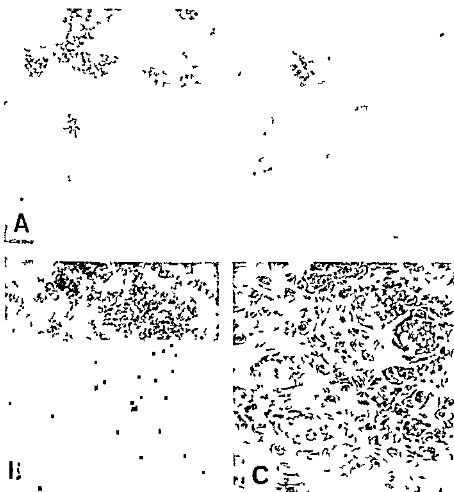


Fig 4 Microautoradiography of superior cervical ganglion from a rat pretreated with guanethidine sulphate 20 mg/kg for 11 days followed by a single injection of ^{3}H guanethidine sulphate 70 mg/kg (a total of 1 2 mci) The animal was killed 24 hours after the injection of labelled guanethidine sulphate Section thickness approximately 8 μ. Exposure time 7 weeks For micro-autoradiography the method of BAY was used (unpublished results)

A unstained specimen magnification x 140 B and C haematoxylin eosin stained specimen magnification x 280 In B the microscope was focused on the film layer in C on the histological specimen

Guanethidine sulphate in doses above 10 mg/kg induced an increase in the ganglionic dry weight (fig 1) due to infiltration of small cells (figs 1 and 5 table 1) The ganglion nerve cells showed chromatolytic changes (fig 5) and a variable number of cells degenerated completely (fig 1, table 1) Following guanethidine sulphate 20 mg/kg for 14 days significant

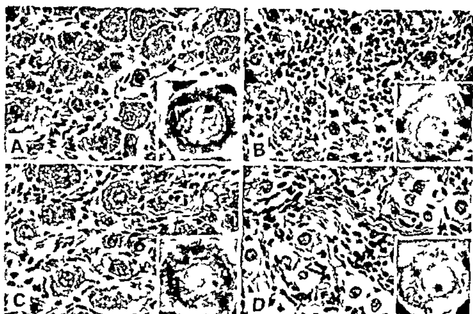


Fig 5 Histological specimens of rat superior cervical ganglia stained by a combination of the gallocyanine chromalun method of EINARSON (1932) and the silver impregnation method of BODIAN (1936) magnification x 190 Inserts of single ganglion cells stained by the gallocyanine chromalun method magnification x 480 A untreated control B pretreatment with guanethidine sulphate 20 mg/kg for 14 days C guanethidine sulphate 60 mg/kg for 14 days with the simultaneous administration of desmethylimipramine chloride 30 mg/kg divided in two daily doses D guanethidine sulphate 20 mg/kg for 14 days with the simultaneous administration of reserpine 0 6 mg/kg daily

were observed *in all three parameters* (*P* $< 0\,001$, *t test*) The degree of irreversible degeneration of the nerve cells based on the these cell counts and weights is graphically presented in another paper (JUUL & MCISAAC 1973)

Simultaneous administration of *DMI* prevented the effects of guanethidine on ganglionic weight and histology (figs 3 and 5 table 1) Simultaneous administration of *reserpine* did not alter the effects of guanethidine on the histology of the ganglia (fig 5 table 1) but the weights of the ganglia were lower (fig 3)

Simultaneous administration of *SKF 525 A* potentiated the effects of guanethidine following guanethidine sulphate 10 mg/kg for 14 days histological changes were observed This was not observed in animals treated only with guanethidine sulphate at this dose SKF 525 A actually inhibited the metabolism of guanethidine at the dose regimens used 12 hours after the discontinuation of guanethidine sulphate 20 mg/kg for 7 days the content of guanethidine in the superior cervical ganglia was 94 ng/ganglion (S E M 9 3 $n = 5$) following identical doses with the simultaneous administration of

Table 1

Cell counts in histological specimens and dry weight of rat superior cervical ganglia following pretreatment of the animals with various drugs Guanethidine sulphate was administered intraperitoneally for 14 days with or without simultaneous intraperitoneal administration of desmethylimipramine hydrochloride (DMI) 30 mg/kg daily divided in two doses or reserpine (RES) 0 6 mg/kg once daily Mean values, S E.M (in brackets), and number of experiments (n) are shown

	Dose of guanethidine sulphate mg/kg	n	Small cells per mm²	Nerve cells per mm²	n	Dry weight µg/ganglion
Control		32	3235 (110)	674 (23)	16	407 (16)
Guanethidine	20	16	5327 (430)	373 (42)	25	620 (20)
Guanethidine + DMI	20	2	4395	640	4	400 (38)
Guanethidine + RES	20	2	5615	365	6	505 (31)
Guanethidine	40	8	5516 (478)	298 (31)	8	671 (47)
Guanethidine + DMI	40	2	4230	675	2	461
Guanethidine	60	4	6093 (794)	218 (28)	4	642 (45)
Guanethidine + DMI	60	2	3790	550	2	400

SKF-525-A the content of guanethidine was 156 ng/ganglion (S E M 7 8, n = 5), the difference being significant (P = 0 01 (Wilcoxon's test))

DMI, reserpine, and SKF-525 A administered alone did not change the histological picture

DMI prevented the sympatholytic effect of guanethidine as judged by the narrowing of the palpebral fissure SKF-525-A did not change the effect of guanethidine in this respect

Discussion

Accumulation of guanethidine by sympathetic ganglia The present investigation demonstrates that guanethidine is concentrated in the sympathetic ganglia as has been shown in peripheral sympathetically innervated organs (BRODIE *et al* 196
OATES 1970, SCHANKER ,
similar accumulation in 1

neurone blocking agents has been shown by other investigators, e g for bretylium (BOURA *et al* 1960), bethanidine (BOURA *et al* 1962), and guanacline (KRONEBERG *et al* 1967)

Localization of ganglionic guanethidine The ganglionic content of guanethidine is lowered considerably by *DMI* indicating that the major part of guanethidine is taken up into the ganglion nerve cells via the membrane pump This uptake mechanism has been shown to occur throughout the entire postganglionic neurone (HAMBERGER *et al* 1964) The intraneuronal localization of guanethidine was confirmed by microautoradiography (fig 4) The uptake of guanethidine in peripheral sympathetically innervated organs described as specific, saturable, and energy dependent is similarly inhibited by tricyclic antidepressants (e g MITCHELL & OATES 1970, SCHANKER & MORRISON 1965)

The mainly intraneuronal localization of guanethidine may explain the diminished content of guanethidine following long term administration of 20–40 mg/kg (fig 1) since an increasing number of ganglion cells was completely degenerated under these conditions (fig 1) as also found by BURNSTOCK *et al* (1971)

Although weighing approximately 10 times more than the rat ganglia, the ganglia of the cat contained only approximately twice the content of guanethidine However, the cat ganglia only contain 3 times as many ganglion cells (BILLINGSLEY & RANSON 1918, KLINGMAN & KLINGMAN 1965)

The blocking effect of DMI on the membrane pump is complicated by its inhibitory effect on the metabolism of guanethidine in the rat (MITCHELL *et al* 1970) The potentiation by SKF-525-A of the histological effects of guanethidine, however, indicates that the parent compound itself possesses cytotoxic effects since the doses of SKF-525-A used at least partially inhibits the metabolism of guanethidine Since DMI completely prevented the histological changes (fig 5, table 1) as well as the sympatholytic effects of guanethidine, the blocking effects of DMI on the amine pump can be considered as almost complete

Whereas the uptake of guanethidine in sympathetic ganglia resembles that of the peripheral organs with regard to the sensitivity to DMI, it differs with regard to its sensitivity to reserpine In the peripheral noradrenergic terminals guanethidine is mainly localized in the noradrenaline storage granules (e g MAÎTRE & STAEHELIN 1971), and reserpine lowers the content of guanethidine in these organs (BRODIE *et al* 1965, CHANG *et al* 1965, MAITRE & STAEHELIN 1971) In the sympathetic ganglia only a small part of guanethidine is sensitive to reserpine This may be explained by different storage mechanisms for guanethidine in the sympathetic ganglia and in the peripheral sympathetic terminals Similar differences in the storage of noradrenaline have been reported, e g by CHUBB *et al* (1972) However, doses of

reserpine 10–20 times higher than those depleting the peripheral stores of noradrenaline do deplete ganglionic noradrenaline (NORBERG 1965) The reason for this discrepancy between the doses of reserpine necessary to deplete noradrenaline in the peripheral and ganglionic storage sites is unknown but it cannot be excluded that the same relation might also be present in the case of guanethidine

Estimation of intraneuronal concentration of guanethidine The rat superior cervical ganglion contains approximately 40,000 nerve cells (KLINGMAN & KLINGMAN 1965) With an average diameter of approximately 30 μ the total nerve cell cytoplasm constitutes 0 5–0 6 μl Based on this figure and the extent of irreversible degeneration of the nerve cells (fig 1, table 1), the concentration of intraneuronal (DMI sensitive) but extra granular (reserpine insensitive) guanethidine may be roughly estimated to be 0 3–0 6 mM following the administration of guanethidine sulphate 20 mg/kg for 14 days This concentration is 10–20 times higher than the concentration of non specifically (DMI insensitive) located guanethidine The intraneuronal concentration may even have been estimated at too low a value owing to the inhibition by DMI of the metabolism of guanethidine resulting in an increased non specific fraction

Relation between the ganglionic content of guanethidine and the selective cytotoxic effects Although guanethidine is concentrated in the peripheral sympathetically innervated organs and in the adrenal gland no structural or ultrastructural changes have been observed in these organs following the doses used in the present investigation (CLEMENTI 1965 JENSEN HOLM & JUUL 1971, JUUL, unpublished results) Very large doses (600 mg/kg) however do produce mitochondrial changes in the adrenal medulla (CLEMENTI 1965) The lower sensitivity to the cytotoxic effects of guanethidine in the peripheral noradrenergic terminals and in the adrenal medulla could be explained by a mainly intragranular storage mechanism protecting' against the toxic effects of guanethidine, whereas a mainly extragranular, "free" localization in the ganglion cells could explain the selective effect in these cells A 50% inhibition of the oxidative phosphorylation in rat liver mitochondria *in vitro* by 0 9 mM guanethidine has been reported by MALMQUIST & OATES (1968) This concentration is not far from that estimated in the present investigation following the administration of doses of guanethidine sulphate which produce morphological changes

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plied by Danish CIBA-GEIGY Dr A M White, CIBA, Horsham, kindly provided the ^{3}H-guanethidine SKF-525-A was supplied by Smith, Kline & French Anne Persson performed the microautoradiographic analysis Annette Francker performed all the remaining analyses

REFERENCES

Billingsley, P R & S W Ranson On the number of nerve cells in the ganglion cervicale superius and of nerve fibers in the cephalic end of the truncus sympathicus of the cat and on the numerical relations of preganglionic and postganglionic neurones *J comp Neurol* 1918, 29, 359–384

Bodian, D A new method for staining nerve fibers and nerve endings in mounted paraffin sections *Anat Rec* 1936, 65 89–97

Boura, A L A, F C. Copp, W G Duncombe, A F Green & A McCoubrey The selective accumulation of bretylium in sympathetic ganglia and their postganglionic nerves *Brit J Pharmacol* 1960, 15, 265–270

Boura, A L. A, W G Duncombe, R D Robson & A McCoubrey The distribution and excretion by cats of a new hypotensive drug N benzyl N N'' dimethylguanidine *J Pharm Pharmacol* 1962, 14, 722–726

Brodie, B B, C C Chang & E Costa On the mechanism of action of guanethidine and bretylium *Brit J Pharmacol* 1965, 25, 171–178

Broen Christensen, C Distribution and biliary excretion of decamethonium in doubly nephrectomized rabbits *Acta pharmacol et toxicol* 1965 23 275–286

Burnstock, G, B Evans, B J Gannon, J W Heath & V James A new method of destroying adrenergic nerves in adult animals using guanethidine *Brit J Pharmacol* 1971, 43 295–301

Chang, C C E Costa & B B Brodie Interaction of guanethidine with adrenergic neurons *J Pharmacol* 1965 147 308–312

Chubb I W, W P De Potter & A F De Schaepdryver The storage of noradrenaline in sympathetic ganglia *Life Sci* 1972, 11 323–333

Clementi, F Modifications ultrastructurelles provoquées par quelques médicaments sur les terminaisons nerveuses adrenergiques et sur la medullaire surrenale *Experientia* 1965, 21 171–176

Einarson L A method for progressive selective staining of Nissl and nuclear substance in nerve cells *Amer J Path* 1932 8 295–308

Furst, C I The biochemistry of guanethidine *Adv Drug Res* 1967, 4 133–161

Furst, C I Studies on the distribution and excretion of a metabolite of guanethidine in the rat. *Brit J Pharmacol* 1968, 32, 57–64

Hamberger, B T Malmfors K A Norberg & Ch Sachs Uptake and accumulation of catecholamines in peripheral adrenergic neurons of reserpinized animals studied with a histochemical method *Biochem Pharmacol* 1964 13, 841–844

Jensen Holm J & P Juul The effects of guanethidine, pre and postganglionic nerve division on the rat superior cervical ganglion Cholinesterases and protein *Acta pharmacol et toxicol* 1970a 28 270–282

Jensen Holm J & P Juul The effects of guanethidine pre and postganglionic nerve division on the rat superior cervical ganglion cholinesterases and catecholamines (histochemistry), and histology *Acta pharmacol et toxicol* 1970b, 28, 283–298

Jensen Holm, J & P Juul Ultrastructural changes in the rat superior cervical ganglion following prolonged guanethidine administration *Acta pharmacol et toxicol* 1971, 30, 308–320

Juul P & R L McIsaac The effect of guanethidine on the noradrenaline content of the adult rat superior cervical ganglion *Acta pharmacol et toxicol* 1973, 32, 382 389

Klingman, G I & J D Klingman Effects of immunosympathectomy on the superior cervical ganglion and other adrenergic tissues of the rat *Life Sci* 1965, 4, 2171 2179

Kroneberg G., K Schlossmann & K Stoepel Zur Pharmakologie von N (2 Guanidinoathyl)-4-methyl 1, 2, 3, 6-tetrahydropyridin, einer neuen antihypertensiv wirkenden Verbindung *Arzneimittelforsch* 1967, 17, 199–207

Maître L & M Staehelin Guanethidine uptake and noradrenaline depletion in noradrenaline storage particles of the rat heart *Biochem Pharmacol* 1971, 20, 1233–1242

Malmquist, J & J A Oates Effects of adrenergic neuronblocking guanidine derivatives on mitochondrial metabolism *Biochem Pharmacol* 1968 17, 1845–1854

Mitchell, J R & J A Oates Guanethidine and related agents I Mechanism of the selective blockade of adrenergic neurons and its antagonsim by drugs *J Pharmacol* 1970 172, 100–107

Mitchell, J R., J H Cavanaugh, J V Gingell & J A Oates Guanethidine and related agents II Metabolism by hepatic microsomes and its inhibition by drugs *J Pharmacol* 1970, 172 108–114

Norberg K A Drug induced changes in monoamine levels in the sympathetic adrenergic ganglion cells and terminals *Acta physiol scand* 1965 65 221–234

Schanker, L S & A S Morrison Physiological disposition of guanethidine in the rat and its uptake by heart slices *Int J Neuropharmacol* 1965 4 27–39

Acta pharmacol et toxicol 1973, 32, 500–512

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Effects of Various Antihypertensive Guanidine Derivatives on the Adult Rat Superior Cervical Ganglion: Histology, Ultrastructure, and Cholinesterase Histochemistry

By

Per Juul

(Received December 22 1972 Accepted January 31, 1973)

Abstract A series of postganglionic neurone blocking agents was administered intraperitoneally in large doses for 2–3 weeks to adult male rats and the effects on the superior cervical ganglion were investigated *Histologically* guanethidine and guanacline induced pronounced changes consisting of chromatolysis of the nerve cells accompanied by an infiltration of small cells A varying number of ganglion cells completely degenerated Guanidine N (ß guanidinoethyl) hexa hydrobenzo-[d] azocine (Ph 881/7) guanoxan debrisoquin bethanidine bretylium and reserpine did not change the histological picture *Ultrastructural changes* of the nerve cells consisting mainly of mitochondrial swelling with partial loss of mitochondrial crests were observed to a major extent following guanethidine and guanacline (with the latter drug furthermore numerous lipofuscin pigment granules appearing in the cytoplasm) and to a minor extent following bretylium debrisoquin bethanidine and guanoxan Guanidine Ph 881/7 and reserpine did not produce any changes *Histochemically demonstrated cholinesterases* were diminished following guanethidine and guanacline mainly from the cytoplasm of the nerve cells The other guanidinium compounds did not produce changes in the cholinesterases No structure activity relationship was established concerning the ganglionic effects of the drugs The changes are considered to be cytotoxic effects of the compounds and the differences in response are assumed to be due to different potency and different extents of accumulation in the sympathetic ganglion nerve cells

Key words Guanidine derivatives ganglia autonomic structure – ultrastructure cholinesterases

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Guanethidine and guanacline have been shown to induce changes in the histology and ultrastructure of adult rat superior cervical ganglia (JENSEN HOLM & JUUL 1970b & 1971 BURNSTOCK *et al* 1971a, ERANKO & ERÄNKÖ 1971b) An almost complete chemical sympathectomy is brought about in

newborn rats by a few injections of guanethidine sulphate 20 mg/kg (ERÄNKÖ & ERÄNKÖ 1971a&b) whereas larger doses for several months are necessary to induce similar effects in the adult rat (BURNSTOCK *et al* 1971b) In newborn animals changes of various types can be induced in the sympathetic ganglia by several compounds antiserum to the nerve growth factor (LEVI-MONTALCINI & ANGELETTI 1966), 6-hydroxy-dopamine (ANGELETTI & LEVI-MONTALCINI 1970), bretylium (CARAMIA *et al* 1972), and reserpine (ANGELETTI & LEVI MONTALCINI 1972)

The purpose of the present investigation has been to study possible effects of various postganglionic neurone blocking agents on the structure, ultrastructure, and cholinesterase histochemistry of the adult rat superior cervical ganglia A series of guanidinium compounds with different chemical formula was selected to study a possible structure-activity relationship with regard to the ganglionic effects The postganglionic neurone blocking agent bretylium was included in the study because of its different chemical constitution, and reserpine was *included to investigate whether the depletion* of catecholamines *per se* would produce structural changes in the sympathetic ganglia

Material and Methods

Male Wistar rats weighing 150–275 g at the termination of the experiments were used Bretylium and reserpine were used in commercially available solutions Ph 881/7 was dissolved in water, the remaining compounds were dissolved in saline. The compounds were administered intraperitoneally once or twice daily for 14 or 21 days The

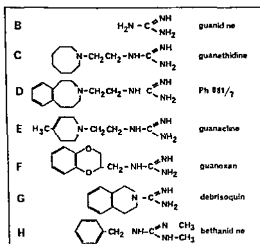


Fig 1 Chemical formulae of the adrenergic neurone blocking guanidine derivatives investigated

Table 1

Cell counts in histological specimens from rat superior cervical ganglia following the administration of various hypotensive drugs at different doses The drugs have been administered for 14 days or 21 days (indicated by *) The figures have been cumulated since no dose response relationship was observed except for guanethidine and guanacline • Some of the figures are taken from JUUL & SAND (1973) n = number of animals For the controls S D is given in brackets

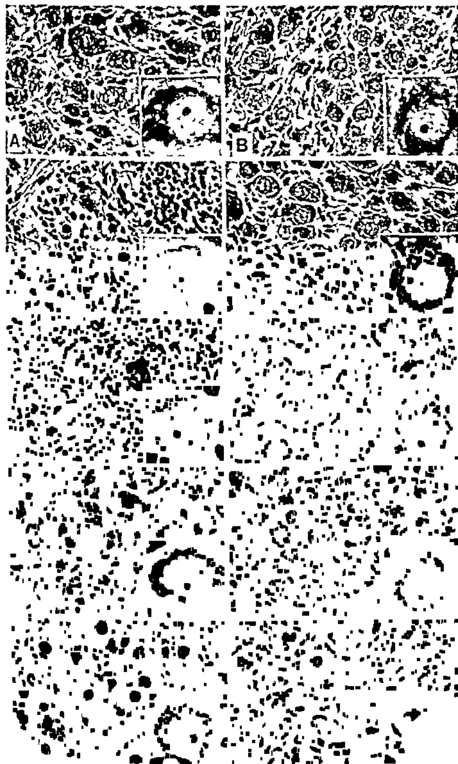
Drug	Doses mg/kg	n	Small cells per mm²	Ganglion cells per mm²	Ratio small cells/ ganglion cells
A Control•		32	3235 (623)	674 (130)	4 9 (0.2)
B Guanidine	20, 40, 60*	5	3216	638	5.2
C. Guanethidine•	20, 40, 60*	26	5207	336	19 1
D Ph 881/7	20, 40, 60*, 80	7	3374	833	4 1
E Guanacline	20, 40, 60*	4	7045	351	126
F Guanoxan	20, 40, 60*, 80	7	3167	776	4 1
G Debrisoquin	40, 60*, 80	6	3305	740	4 5
H Bethanidine	60*, 100, 120, 150	4	3629	828	4 3
I Bretylium	20, 40	3	3902	825	4 9
J Reserpine	0 3, 0 5, 1 0	4	3224	699	4 7

rats were killed by exsanguination under ether anaesthesia 1 or 2 days following discontinuation of administration The superior cervical ganglia were excised and prepared for histology, electron microscopy, or cholinesterase histochemistry as described previously (JENSEN HOLM & JUUL 1970b & 1971) For cholinesterase histochemistry acetylthiocholine was used as substrate, and the pre incubation and incubation was carried out at 4-8° Cell counts were performed on microphotographs comprising an area of 0 1 mm² in the controls and 0 2 mm² in the treated animals (2 or 4 different visual fields per ganglion at 400 × magnification were observed) A Leitz Orthoplan microscope equipped with an Orthomat camera was used for histology and histochemistry, and an AEI "Corinth" was used for the study of ultrastructure

Appart from the control animals which received saline, the following drugs were administered

A Control
B Guanidine hydrochloride (SIGMA)
C Guanethidine sulphate (ismelin® CIBA)
D N (β guanidinoethyl) hexahydrobenzo [d] azocine sulphate (Ph 881/7 Pharmacia)

Fig 2 The histological picture of rat superior cervical ganglia following the administration for 21 days at 60 mg/kg of the compounds B I stated in Material and Methods and in fig 1 The specimens were stained by a combination of the gallocyanine-chromalun method of EINARSON (1932) and the silver impregnation technique of BODIAN (1936) magnification × 190 Inserts single nerve cells stained by the gallocyanine chromalun method, magnification × 480


A
B

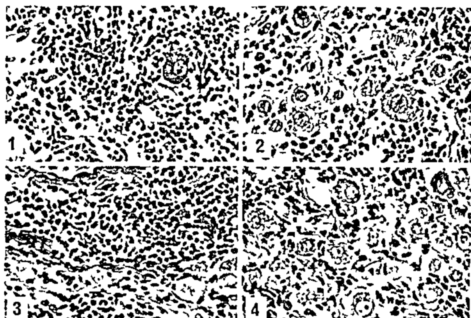


Fig 3 Variations in histological response in superior cervical ganglia from different rats treated with identical doses (60 mg/kg for 21 days) of guanethidine (1–2) and guanacline (3–4) Staining procedure as in fig 2. Magnification × 190

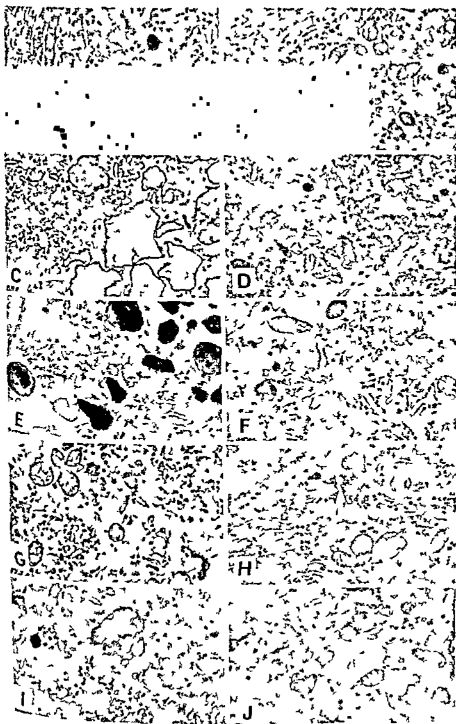
E Guanacline sulphate (cyclazenine, leron® Bayer)
F Guanoxan sulphate (envacar® Pfizer)
G Debrisoquin sulphate (isocaramidine, declinax® Roche)
H Bethanidine sulphate (regulin® GEA)
I Bretylium tosylate (darenthin® Wellcome)
J Reserpine (serpasil® CIBA)

The chemical formulae of the guanidine derivatives are shown in fig 1 The letters refer to the microphotographs and electron micrographs in the figures The doses are stated in mg/kg of the salts With regard to the guanidinium compounds a dose of 60 mg/kg corresponds to approximately 20 (17–27) × 10^{-5} mol per kg

The doses used in the study of *histology* are shown in table 1 For *electron microcopy* were used ganglia from 2 or 3 rats treated with different doses guanidine 40 and 60 mg/kg, guanethidine 20 40 and 60 mg/kg, Ph 881/7 60 and 80 mg/kg, guanacline 40 and 60 mg/kg, guanoxan 60 and 80 mg/kg, debrisoquin 40, 60, and 80 mg/kg, bethanidine 60, 120, and 150 mg/kg bretylium 16 and 40 mg/kg, reserpine 0 3 and 1 0 mg/kg For the study of *cholinesterase histochemistry* the animals received 60 mg/kg daily of the guanidinum compounds

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Fig 4 Ultrastructure of rat superior cervical ganglia following the administration for 21 days at 60 mg/kg of the compounds B H, bretylium (I) 40 mg/kg, and reserpine (J) 1 mg/kg for 14 days Magnification × 16,000


C
D
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G
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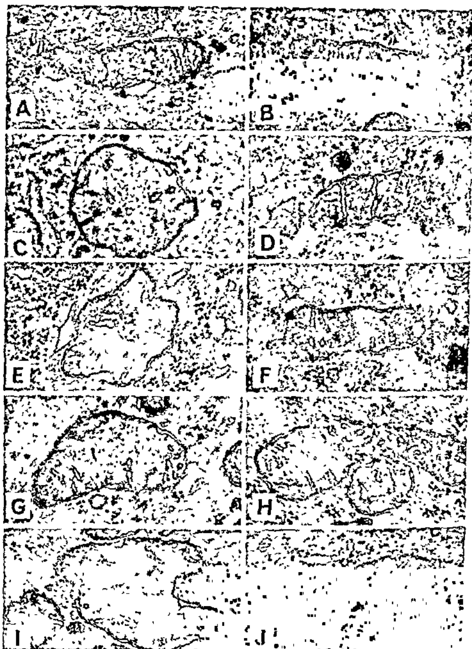


Fig 5. Ultrastructure of rat *superior* cervical ganglion treated with compounds as in fig 4 Magnification × 39,000.

Results

I Histology

The histological pictures obtained with the various compounds are shown in fig 2, the cell counts in table 1. *Guanethidine* and *guanacline* at the doses used induced chromatolysis of the nerve cells accompanied by an infiltration of small cells Some of the nerve cells completely degenerated Within the individual ganglion the small cells were often located in "lumps", perivascular "collars", or subcapsularly at the periphery of the ganglion Within the ganglia from different animals a considerable variation in response to identical doses was observed An almost complete disappearance of all ganglion cells could be observed in some specimens following large doses of both drugs (fig 3)

The other compounds tested did not induce any histological changes In table 1 the cell counts obtained from animals treated at different dose levels are cumulated since only qualitative effects were looked for

II Ultrastructure

Guanacline induced the most profound ultrastructural alterations Following 40–60 mg/kg for 14–21 days the changes were dominated by the occurrence of numerous dense bodies, probably lipofuscin pigment granules in the cytoplasm of the ganglion nerve cells The mitochondria were dilated with partial loss of cristae (fig 4–5 E)

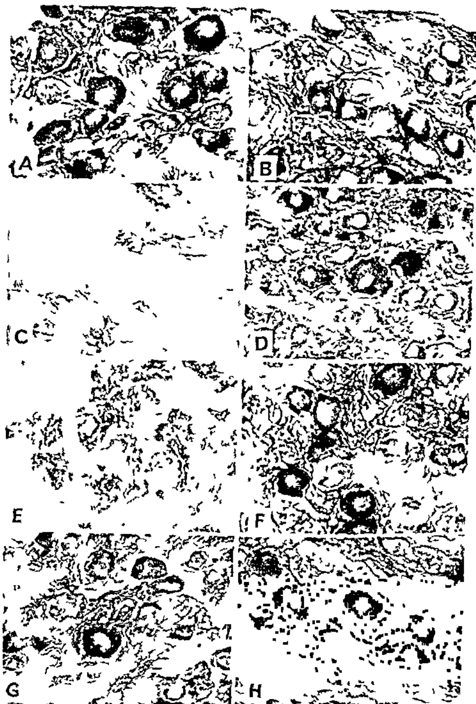
Guanethidine at similar doses induced severe changes in the nerve cells, the dominant lesion consisting of enormous dilatation of the mitochondria with rupture and loss of cristae, but with preserved outer membranes No accumulation of lysosomes or lipofuscin granules was observed (fig 4–5 C)

Guanoxan, debrisoquin, bethanidine, and bretylium at the doses stated in Material and Methods induced minor and variable swelling of the mitochondria with partial loss of cristae (fig 4–5 F,G,H,I) Based on a subjective evaluation of the severity of the changes the drugs may be listed in the following order bretylium > debrisoquin > bethanidine > guanoxan No changes in the mitochondria of other cells in the sympathetic ganglia were observed following the drugs mentioned above

Guanidine, Ph 881/7, and *reserpine* did not induce any changes at the doses used (fig 4–5 B,D,J)

III Cholinesterase histochemistry

Guanethidine and *guanacline* administered at 60 mg/kg for 21 days induced an almost complete loss of the cholinesterase activity from the cytoplasm of the nerve cells and a varying loss of the activity in the preganglionic nerve fibers (fig 5 C,E) None of the other drugs induced changes in the cholinesterase histochemistry


A
B
C
D
E
F
G
H

At the doses used all the compounds except guanidine induced narrowing of the palpebral fissure

Discussion

Prolonged administration of guanethidine at 20 mg/kg has been shown to induce pronounced changes in the sympathetic ganglia of adult rats consisting of chromatolysis of the nerve cells accompanied by an infiltration of small cells, with the electron microscope a tremendous dilatation of the mitochondria with partial loss of cristae was observed, the cholinesterase activities were lowered, and the noradrenaline content was diminished (JENSEN HOLM & JUUL 1970a, b & 1971, JUUL & McISAAC 1973) The changes in cholinesterases and noradrenaline content were assumed to be secondary to the chromatolysis Prolonged administration of guanacline at 5 mg/kg also produced severe changes in the sympathetic ganglia, but the ultrastructure was dominated by the appearance of numerous lipofuscin pigment granules in the cytoplasm of the nerve cells (BURNSTOCK *et al* 1971a) In the present investigation the changes produced by the two drugs are in agreement with the previous findings Furthermore guanacline was shown to induce changes in histology and cholinesterase histochemistry similar to those resulting from guanethidine treatment It should be noted that the doses of guanethidine and guanacline used in some of the present experiments are higher than in previous investigations (JENSEN HOLM & JUUL 1970a, b & 1971, BURNSTOCK *et al* 1971a & b, ERANKO & ERANKO 1971b) Thus the disappearance of cholinesterase activity from the nerve cell body was to be expected It is reasonable to assume that the loss of cholinesterases results from the chromatolysis as is the case following postganglionic axotomy (see JENSEN HOLM & JUUL 1970b) and following chemical sympathectomy by 6 hydroxydopamine (ERANKO & ERÄNKO 1972)

Some of the other drugs investigated in the present study (bretylium, debrisoquin bethanidine, and guanoxan) induced slight to moderate changes in the ultrastructure (mitochondrial swelling with partial disruption of cristae) without any changes in the histology or the cholinesterase histochemistry The remaining compounds (guanidine, Ph 881/7, and reserpine) did not produce changes in any of the parameters Although the ultrastructural picture differed between guanethidine and guanacline treated animals

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Fig 6 Cholinesterase histochemistry of rat superior cervical ganglia following administration for 21 days at 60 mg/kg of the compounds B H stated in Material and Methods and in fig 1 A modification of the method of KARNOVSKY & ROOTS (1964) using acetylthiocholine as substrate was used (JENSEN HOLM & JUUL 1970b) Magnification × 200

it is possible that the differences between the morphological effects induced by these compounds are only quantitative

The compounds investigated in the present study were selected because of variations in all three parts of the molecule (the ring system, the length of the side chain, and the substitution at the guanidine group, fig 1), but no apparent structure-activity relationship concerning the cytotoxic effects was observed Similarly no correlation was observed between the cytotoxic effect and other effects of the drugs, e g the adrenergic blocking effect, the depletion of tissue noradrenaline, and the inhibition of the oxidative phosphorylation (see PETTINGER & HORST 1971)

Mitochondrial swelling *per se* is an unspecific effect produced by many drugs (e g survey by ROUILLER 1960) and the selective changes in the sympathetic ganglion cells induced by some of the postganglionic neurone blocking agents are probably due to selective accumulation in these cells In a previous paper (JUUL & SAND 1973) it was assumed that the selective cellular effect of guanethidine on the sympathetic ganglion cells was due to high intraneuronal, but extragranular concentrations of the drug Apart from different cytotoxic potencies, however, the compounds may have different kinetics – especially concerning the concentrations obtained in the sympathetic neurones The postganglionic neurone blocking agents are known or assumed to concentrate in the sympathetic neurones but only a few comparable data exist in the literature However, much higher concentrations of guanacline are reached in the sympathetic ganglia than of guanethidine given in similar doses to cats (KRONEBERG *et al* 1967, JUUL & SAND 1973) Such differences in the ganglionic concentrations may help to explain the differences in the cytotoxic effects observed

Even high concentrations of guanethidine added to the medium failed to affect the sympathetic nerve cells in cultures of ganglia from newborn rats (ERANKO *et al* 1972) suggesting the presence of toxic metabolites The various guanidine derivatives are metabolized to different extents which may also be of importance in this respect

The interpretation of the changes is complicated by various sequential phases of the effects (HEATH *et al* 1972), by different sensitivities of different parts of the sympathetic nervous system (EVANS *et al* 1972), and by the variations in response within individual animals and within individual ganglia (fig 3)

The differences in sensitivity between newborn and adult animals to the ganglionic effects of guanethidine (ERANKO & ERANKO 1971b) also apply to the other compounds producing a chemical sympathectomy or immuno-sympathectomy as mentioned in the introduction This difference may reflect a different degree of maturation of the biological membranes or of the cellular metabolism of the sympathetic neurones Furthermore different kinetics of

the drugs in newborn animals cannot be excluded, e g slower hepatic biotransformation or renal excretion

Depletion of catecholamines *per se* did not induce morphological alterations in the ganglion cells since no changes were observed following reserpine This drug induced mitochondrial abnormalities in the dog myocardium (WILCKEN *et al* 1967)

The mechanism underlying the structural effects is unknown but it seems likely that an interference with energy metabolic processes is the primary action Thus the oxidative phosphorylation of rat liver mitochondria *in vitro* is partially inhibited by the guanidine derivatives (MALMQUIST & OATES 1968) at concentrations which may be reached with the high doses used in the present and in other investigations

The adrenergic blocking effect of the drugs can be produced by doses which do not induce morphological changes This does not exclude a continuum between the pharmacological and toxic effects However, evidence linking the cytotoxic effects with the blockade of the noradrenergic transmission is lacking

Acknowledgements

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REFERENCES

Angeletti P U & R Levi Montalcini Sympathetic nerve cell destruction in newborn mammals by 6-hydroxydopamine *Proc Nat Acad Sci* 1970 65 114–121

Angeletti P U, R Levi Montalcini & F Caramia Structural and ultrastructural changes in developing sympathetic ganglia induced by guanethidine *Brain Res* 1972 43 515–525

Angeletti P U & R Levi Montalcini Growth inhibition of sympathetic cells by some adrenergic blocking agents *Proc Nat Acad Sci* 1972 69 86–88

Bodian, D A new method for staining nerve fibers and nerve endings in mounted paraffin sections *Anat Rec* 1936, 65 89–97

Burnstock, G A E Doyle B J Gannon J F Gerkins T Iwayama & M L Mashford Prolonged hypotension and ultrastructural changes in sympathetic neurones following guanacline treatment *Eur J Pharmacol* 1971a 13 175–187

Burnstock G B Evans B J Gannon J W Heath & V James A new method of destroying adrenergic nerves in adult animals using guanethidine *Brit J Pharmacol* 1971b 43 295–301

Caramia F P U Angeletti R Levi Montalcini & L Carratelli Mitochondrial lesions of developing sympathetic neurons induced by bretylium tosylate *Brain Res* 1972 40 237–246

Einarson, L. A method for progressive selective staining of Nissl and nuclear substance in nerve cells *Amer J. Path* 1932, 8, 295–308

Eranko, O & L Eranko Histochemical evidence of chemical sympathectomy by guanethidine in newborn rats *Histochem J* 1971a, 3, 451–456

Eranko L & O Eranko Effect of guanethidine on nerve cells and small intensely fluorescent cells in sympathetic ganglia of newborn and adult rats *Acta pharmacol et toxicol* 1971b, 30 403–416

Eranko L & O Eranko Effect of 6 hydroxydopamine on the ganglion cells and the small intensely fluorescent cells in the superior cervical ganglion of the rat *Acta physiol scand* 1972, 84, 115–124

Eranko L., C Hill, O Eranko & G Burnstock Lack of toxic effect of guanethidine on nerve cells and small intensely fluorescent cells in cultures of sympathetic ganglia of newborn rats *Brain Res* 1972, 43, 501–513

Evans, B, B J Gannon, J W Heath & G Burnstock Longlasting damage to the internal male genital organs and their adrenergic innervation in rats following chronic treatment with the antihypertensive drug guanethidine *Fertility and Sterility* 1972, 23, 657–667

Heath, J W, B K Evans B J Gannon, G Burnstock & V B James Degeneration of adrenergic neurons following guanethidine treatment an ultrastructural study *Virchows Arch Abt B Zellpath* 1972, 11 182–197

Jensen Holm, J & P Juul The effects of guanethidine, pre and postganglionic nerve division on the rat superior cervical ganglion cholinesterases and protein *Acta pharmacol et toxicol* 1970a 28 270–282

Jensen Holm, J & P Juul The effects of guanethidine, pre and postganglionic nerve division on the rat superior cervical ganglion cholinesterases and catecholamines (histochemistry) and histology *Acta pharmacol et toxicol* 1970b,28, 283–298

Jensen Holm, J & P Juul Ultrastructural changes in the rat superior cervical ganglion following prolonged guanethidine administration *Acta pharmacol et toxicol* 1971, 30 308–320

Juul, P & R L McIsaac The effect of guanethidine on the noradrenaline content of the adult rat superior cervical ganglion *Acta pharmacol et toxicol* 1973 32 382–389

Juul, P & O Sand Determination of guanethidine in sympathetic ganglia *Acta pharmacol et toxicol* 1973, 32 487–499

Karnovsky, M J & L Roots A "direct coloring' thiocholine method for cholinesterases *J Histochem Cytochem* 1964, 12 219–221

Kroneberg G K Schlossmann & K Stoepel Zur Pharmakologie von N (2 Guanidinoathyl)-4 methyl 1 2 3 6 tetrahydropyridin, einer neuen antihypertensiv wirkenden Verbindung *Arzneimittelforsch* 1967 17 199–207

Levi Montalcini, R & P U Angeletti Immunosympathectomy *Pharmacol Rev* 1966 18, 619–628

Malmquist J & J A Oates Effects of adrenergic neuronblocking guanidine derivatives on mitochondrial metabolism *Biochem Pharmacol* 1968, 17, 1845–1854

Pettinger, W A & W D Horst Quantifying metabolic effects of antihypertensive and other drugs at the sympathetic neuron level Clinical and basic correlations *Ann N Y Acad Sci* 1971, 179, 310–321

Rouiller, C Physiological and pathological changes in mitochondrial morphology *Int Rev Cytol* 1960, 9, 227 292

Wilcken, D E. L, D Brender, C D Shorey & G J Macdonald Reserpine Effect on structure of heart muscle *Science* 1967 157 1332–1334

Acta pharmacol et toxicol 1973, 32, 513–518

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Reversal by Promazine of Acetylcholine-Induced Changes in Rat Atrial Action Potentials

By

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Abstract In isolated electrically stimulated left rat atria, the application of acetylcholine (ACh) 5×10^{-6} M caused a pronounced shortening of the action potential without changing the resting membrane potential The times required to reach [illegible] % of the [illegible] 94 8 % [illegible] was increased to 105 2 % ($0 1 > P > 0 05$) The addition of promazine 5×10^{-6} M and 10^{-5} M to atria pretreated with ACh increased the duration of the action potential and thus reversed the effects of ACh The effects were most marked after promazine 10^{-5} M, which prolonged 50 and 90 % repolarization times to 135 8 ($P < 0 001$) and 140 0 % ($P < 0 001$) of the initial values and reduced dv/dt to 71 2 % ($P < 0 001$) The height of the action potential was reduced by promazine 10^{-5} M to 94 9 % ($P < 0 02$) of the initial value, this being the only indication of a similarity of effect between these two cardio-depressive agents The alterations in ionic fluxes underlying the changes in electrical activity are briefly discussed

Key words Promazine – anticholinergic effects – rat atria – action potential

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We have previously reported that promazine and thioridazine, two phenothiazines with cardio-depressive effects (LANDMARK 1971a) reduce the inhibitory effects of acetylcholine on rat atria (HAFFNER & LANDMARK 1972) The anticholinergic action of the phenothiazines appeared to be unspecific, i e not due to receptor-blockade, which suggested that it could be due to opposing effects of acetylcholine and the phenothiazines on cell membrane permeability If this suggestion is correct, the phenothiazines should be able to counteract the shortening in membrane action potential produced by acetylcholine (ANTONI & ROTMANN 1968), since this shortening is believed to be due to a change in membrane permeability

The present study was undertaken to determine whether the interactions between acetylcholine and the phenothiazines previously described could

be explained by the effects of these drugs on electrical activity We have studied the effects of acetylcholine on the resting membrane and on the action potential in isolated electrically stimulated rat atria and have attempted to reverse the effects of acetylcholine by adding promazine

Material and Methods

Female albino Wistar rats weighing approximately 200 g were used The left atria were prepared mounted and stimulated as described previously (LANDMARK 1971b) The stimulus frequency used was 180/min

When the atria had been suspended in the organ bath for approximately 5 min recordings of the membrane action potentials were taken over the next 30 min (control period) the drug to be tested was then added to the Ringer solution perfusing *the organ bath and recordings were taken for a further 30 min (test period)*

The recordings and measurements of action and membrane potentials were carried out as described previously (LANDMARK 1971b) The parameters studied were resting potential the height of the action potential, the maximum rate of rise of the action potential (V/sec) and the time for 50 and 90 per cent repolarization

Drugs used Fresh solution of promazine HCl (Norfarma) in deionized water were made each day and kept in the dark at 4° The drug was added in amounts which gave final bath concentrations of 5×10^{-6} M and 10^{-5} M Acetylcholine chloride solutions were prepared from a frozen stock solution, and added in amounts which gave a concentration of 5×10^{-6} M in the bath

Results

Acetylcholine $5 \times 10^{-6} M$ had no effect on the resting membrane potential, but markedly altered the action potential (table 1) The most obvious effect was the increased rate of repolarization, 50 and 90 % repolarization were reached nearly twice as fast after treatment with acetylcholine The height of the action potential (greatest depolarization) was reduced and the maximal rate of rise was slightly increased, but these changes were not statistically significant ($0\,1 > P > 0\,05$)

When acetylcholine was added right at the start of the experiments (table 2) and the values for the control period were compared with those for the test period, there was a slight increase in the effects with time of exposure This change had to be taken into account when the effect of promazine were studied, and it was therefore necessary to convert all the values into per cent of the mean values for the control period, in order to compare the changes in the different preparations

Promazine $5 \times 10^{-6} M$ caused a statistically significant reduction in the rate of repolarization in atria pretreated with acetylcholine, but had no

Table 1

Effects of acetylcholine (ACH) on intracellularly recorded rat atrial potentials Mean values ± S E M The percentages are calculated from the initial mean (control 1 and control ACH) in the same preparations. n = number of observations, in brackets number of atria. Significance of difference between test and control periods evaluated by Student s t test

	Resting potential −mV	Action potential mV	Max rate of rise V/sec	50 % repol msec	90 % repol msec
Control 1 *	59 7 ± 0 4	63 9 ± 0 5	107 2 ± 4 3	10 2 ± 0 4	37 6 ± 1 2
n (4)	159	159	87	74	65
Control 2 *	57 4 ± 1 1	62 4 ± 0 6	99 3 ± 5 0	9 8 ± 8 0	37 4 ± 1 6
n	146	146	82	62	46
Control 2 in %	96 1 ± 1 84	97 7 ± 0 94	92 6 ± 4 66	96 1 ± 3 92	99 5 ± 4 26
Control ACH	58 02 ± 0 52	65 0 ± 0 57	103 67 ± 9 55	12 37 ± 0 47	32 85 ± 1 13
n (4)	200	200	95	104	86
ACH 5×10^{-6} M	57 51 ± 0 66	61 65 ± 0 78	109 01 ± 5 23	6 49 ± 0 33	15 72 ± 0 9
n	161	160	70	90	69
ACH 5×10^{-6} M in %	99 1 ± 1 14	94 8 ± 1 2	105 2 ± 5 04	52.5 ± 2 67	47 9 ± 2 74
Change due to ACH in %	+3 0	−2 9	+12 6	−43 6	−51 6
Significance	P < 0.2	P < 0 1	P < 0 1	P < 0 001	P < 0 001

* From LANDMARK 1971b

statistically significant effect on the height or the maximal rate of rise of the action potential, nor did it affect the resting membrane potential (table 2)

Promazine 10^{-5} M caused an even greater reduction in the rate of repolarization, and also significantly reduced the maximum rate of rise This concentration of promazine also produced a slight, but statistically significant ($P < 0\,02$) *reduction in the height of the action potential* (table 2)

Compared with the control atria untreated by drugs it will be seen that both promazine 5×10^{-6} M and 10^{-5} M fail to reduce the rate of repolarization by as much as acetylcholine increases it, i e the effect of acetylcholine is not fully reversed The maximal rate of rise on the other hand, while only slightly increased by acetylcholine, was greatly reduced by promazine 10^{-5} M, the effect of acetylcholine was more than reversed, final values being far lower than those in preparations untreated with drugs It

Table 2

Effects of promazine (PR) on intracellularly recorded electrical potentials in isolated rat atria treated with acetylcholine (ACH) Mean values ± S E M The percentages are calculated from the initial mean in the same preparations n=number of observa tions in brackets number of atria Significance of difference between test and control periods evaluated by Student s t test

	Resting potential −mV	Action potential mV	Max rate of rise V/sec	50 % repol msec	90 % repol. msec
ACH 5×10^{-6} M 1st	59 5 ± 0 55	69 43 ± 0 65	119 08 ± 4 02	8 72 ± 0 2	19 7 ± 0 37
n (4)	298	297	141	153	146
ACH 5×10^{-6} M 2nd	58 94 ± 1 95	68 37 ± 0 63	123 91 ± 4 63	7 11 ± 0 15	17 4 ± 0 32
n	291	290	131	150	146
2nd ACH in %	99 1 ± 3 3	98 5 ± 0 91	104 1 ± 3 89	81 5 ± 1 72	88 3 ± 1 62
ACH 5×10^{-6} M	58 79 ± 0 46	67 98 ± 0 75	102 88 ± 4 71	6 67 ± 0 15	16 44 ± 0 35
n (3)	211	210	100	111	104
ACH 5×10^{-6} + PR 5×10^{-6} M	57 66 ± 0 56	66 13 ± 0 62	104 84 ± 10 37	7 64 ± 0.22	19 68 ± 0 57
n	191	189	94	98	94
ACH + PR 5×10^{-6} in %	98 1 ± 0 95	97 3 ± 0 91	101 9 ± 10 08	114 5 ± 3 3	119 7 ± 3 47
Change due to PR 5×10^{-6} in %	−1 0	−1 2	−2 2	+33 0	+31 4
Significance	P < 0 8	P < 0 7	P < 0 9	P < 0 001	P < 0 001
ACH 5×10^{-6} M n (4)	58 85 ± 0 42	63 23 ± 2 13	109 55 ± 6 3	8 08 ± 0 21	21 07 ± 0 49
	274	273	126	147	133
ACH 5×10^{-6} + PR 10^{-5} M	57 5 ± 0 53	60 05 ± 0 73	78 03 ± 2 88	10 97 ± 0 36	29 68 ± 0 87
n	274	271	119	154	132
ACH+PR 10^{-5} in %	97 7 ± 0 9	94 9 ± 1 15	71.2 ± 2 63	135 8 ± 4 46	140 0 ± 4 13
Change due to PR 10^{-5} in %	−1 4	−3 6	−32 9	+54 3	+51 7
Significance	P < 0 6	P < 0 02	P < 0 001	P < 0 001	P < 0 01

should also be noted that the height of the action potential was affected *in the same way* by acetylcholine and promazine, a slight reduction occurred on addition of acetylcholine, and a further reduction was produced by promazine

Discussion

The application of ACh to the isolated rat atrium caused a shortening of the action potential, but no hyper-polarization These results are in accordance with previous observations in rat atria (WEBB & HOLLANDER 1956), and corresponding changes have been found in cat and rabbit atria (BURGEN & TERROUX 1953, VAUGHAN WILLIAMS 1959) It has also been shown that ACh causes very little hyper-polarization although the contractile force is inhibited (HOFFMAN & SUCKLING 1953, HUTTER & TRAUTWEIN 1956), the negative inotropic effect is therefore believed to be entirely due to the shortening of the action potential (ANTONI & ROTMANN 1968) In the present study acetylcholine was found to affect all parts or the action potential Although only the reductions in 50 and 90 % repolarization time were statistically significant ($P < 0\ 001$), a reduction in the height of the action potential and an increase in the maximum rate of rise (dv/dt) of depolarization were also observed, but these changes are of borderline significance ($0\ 1 > P > 0\ 05$)

The change in the action potential produced by promazine have been described previously (LANDMARK 1971b) In a concentration of 10^{-5}M, the drug reduced dv/dt and the overshoot of the action potential, without significantly altering the resting membrane potential The drug also caused a slight reduction in the rate of repolarization The results presented above show that promazine 5×10^{-6} M and to a greater extent 10^{-5} M can also reverse the shortening of the action potential caused by ACh, which might explain the anticholinergic effect on contractile force

The ACh induced shortening of the action potential is associated with a specific increase in K^{+} permeability and efflux from atrial muscle (HOLLAND *et al* 1952 BURGEN & TERROUX 1953, HARRIS & HUTTER 1956, HOLLAND *et al* 1959) Promazine, on the other hand, reduces K^{+}-efflux even in a concentration of 5×10^{-6} M, which does not decrease contractile force (LANDMARK *et al* 1972) This may explain the opposing effects of acetylcholine and promazine on the action potential

The change in the action potential produced by promazine have been dewith a reduced uptake of calcium into the myocardium (HODITZ & LÜLLMANN 1964, GROSSMAN & FURCHGOTT 1964) It has also been suggested that the phenothiazine induced depression in contractile force is due to an

interference with the transfer and exchange of calcium available for contraction (LANDMARK 1972) This could explain why both ACh and promazine reduce contractile force.

REFERENCES

Antoni, H & M Rotmann Zum Mechanismus der negativ inotropen Acetylcholin Wirkung auf das isolierte Froschmyokard *Pflügers Archiv* 1968, 300, 67–86

Burgen, A S V & K G Terroux On the negative inotropic effect in the cat's auricle *J Physiol* (Lond) 1953, 120, 449–464

Grossman, A & R F Furchgott The effect of various drugs on calcium exchange in the isolated guinea pig left auricle *J Pharmacol Exp Therap* 1964, **145**, 162–172

Haffner, J F W & K Landmark Opposing anticholinergic and cardiodepressive effects of promazine and thioridazine in isolated rat atria *Acta pharmacol et toxicol* 1972, 31, 529–539

Harris, E J & O F Hutter The action of acetylcholine on the movements of potassium ions in the sinus venosus of the heart *J Physiol (Lond)* 1956, 133, 58–59 P

Hoditz, H & K Lullmann Der Einfluss von Acetylcholin auf den Calciumumsatz ruhender und kontrahierender Vorhofsmuskulatur *in vitro* *Experientia* (Basel) 1964, 20, 279

Hoffman, B F & E E Suckling Cardiac cellular potentials Effect of vagal stimulation and acetylcholine *Amer J Physiol* 1953, **173**, 312–320

Holland, W C, C E Dunn & M E Greig Studies on permeability VIII Role of acetylcholine metabolism in the genesis of the electrocardiogram *Amer J Physiol* 1952, **170**, 339–345

Holland, W C, R L Klein & A H Briggs Factors affecting the action of acetylcholine on transmembrane flux of K in isolated rabbit atria *Amer J Physiol* 1959, **196**, 478–482

Hutter, O F & W Trautwein Vagal and sympathetic effects on the pacemaker fibers in sinus venosus of the heart *J gen Physiol* 1956, 39, 715–733

Landmark, K The action of promazine and thioridazine in isolated rat atria I Effects on automaticity, mechanical performance, refractoriness and excitability *Eur J Pharmacol* 1971a, 16, 1–7

Landmark, K Changes in rat atrial action potential induced by promazine and thioridazine *Acta pharmacol et toxicol* 1971b, 30, 465–479

Landmark, K Changes in rat atrial action potentials induced by promazine and thioridazine of varying concentrations of calcium and different frequencies and strengths of stimulation on contractile force and excitability *Eur J Pharmacol* 1972, 17, 365–374

Landmark, K, J F W Haffner & J Setekleiv Reduction in ^{42}K efflux from rat atria by promazine and thioridazine *Acta pharmacol et toxicol* 1972, 31, 54–64

Vaughan Williams, E M Simultaneous measurements of contractions and intracellular potentials in isolated rabbit atria exposed to acetylcholine *J Physiol* (Lond) 1959, 147, 325–332

Webb, J L & P B Hollander The action of acetylcholine and epinephrine on the cellular membrane potentials and contractility of rat atrium *Circulation Res* 1965, 4, 332–336

Acta pharmacol et toxicol 1973, 32, 519–524

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Actinomycin D and Development of Tolerance to Morphine Analgesia in Rats

By

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Abstract Adult male (Wistar) rats were given an analgesic dose of morphine (10 mg/kg subcutaneously) with or without actinomycin D (10 μg/kg intra peritoneally) daily over a period of 3 or 4 days The degree of analgesia was measured on the hot plate after each injection or after the last injection only A high degree of tolerance to morphine analgesia was observed after only one or two doses of morphine whether actinomycin D was given or not Tolerance developed at about the same rate whether the animals were tested each day on the hot plate or on the last day only Actinomycin D had no analgesic action of its own but it reduced the analgesic action of morphine in animals tested on the first day

Key words Morphine – analgesia – actinomycin D – tolerance – hot plate – behavioural tolerance

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COHEN *et al* (1965) reported that repeated administration of low doses of actinomycin D (meractinomycinum NFN) (10 μg/kg intraperitoneally) inhibits the development of tolerance to morphine analgesia in rats (and mice) Similar results were obtained by COX *et al* (1968) when morphine and actinomycin D (10 μg/kg/hr) were infused simultaneously into rats On the basis of these and other studies on drugs that inhibit RNA and protein synthesis, COX & OSMAN (1970) concluded that the synthesis of new RNA and protein in the brain is an essential feature of the development of tolerance to morphine analgesia in rats However, in experiments involving infusion of morphine into rats, JÓHANNESSON *et al* (1972) found that the administration of morphine in amounts sufficiently high to induce tolerance did not result in any changes in the content of DNA, RNA and protein in the isolated nuclei or in whole brain The investigation reported here was therefore initiated in order to test the validity of the hypothesis

that actinomycin D prevents the development of tolerance to morphine analgesia.

Other investigators (KAYAN *et al* 1969; KAYAN & MITCHELL 1969) have reported that the rate of tolerance development to morphine analgesia as measured by the hot plate method is influenced by the animals' previous experience with the testing procedure. In this investigation, therefore, the effect of actinomycin D was studied both in rats that received daily injections of morphine followed by testing on the hot plate and in rats that received morphine daily but were not tested on the hot plate until on the last day of the experiment.

Methods

Animals

Adult male Wistar (SPF) rats (260-370 g) were used for the experiments The animals were maintained in constant environmental conditions with free access to a commercial food preparation of controlled quality and tap water.

Treatments

Morphine chloride, Ph Nord, was dissolved in distilled water and injected subcutaneously in amounts of 10 mg/kg in a volume of 2 ml/kg Injections were made in the middle of the back at the midline Actinomycin D (Lyovac Cosmegen) was dissolved in distilled water and injected intraperitoneally in amounts of 10 µg/kg (2 ml/kg) in the middle of the abdomen at the midline When both drugs were given to the same animal, morphine was given first and actinomycin D immediately afterwards Actinomycin D, when given alone, did not have any analgesic effect in these experiments

The degree of analgesia was measured by using the hot plate procedure as described by JÓHANNESSON & WOODS (1964) The animals were tested 30 minutes before the injections, just before the injections, and then at 30, 60, 90, and 120 minutes after the injections

Five groups of experimental animals were used

Group I (10 animals) Morphine and actinomycin D injected daily for 4 days followed by analgesimetric testing on each day

Group II (10 animals) Morphine and actinomycin D injected daily for 4 days followed by analgesimetric testing *on the last day only*

Group III (6 animals) Morphine injected daily for 3 days followed by analgesimetric testing on each day

Group IV (10 animals) Morphine injected daily for 4 days followed by analgesimetric testing on each day

Group V (8 animals) Morphine injected daily for 4 days followed by analgesimetric testing *on the last day only*

Rats in groups I, II and III were experimented on first Rats in the two last groups were taken into experiment in order to further substantiate the effect of repeated testing (on the hot plate) in the development of tolerance to morphine analgesia

Statistical analyses were performed by student's grouped "t" test (GOLDSTEIN 1964) The level of significance was taken as $P \leq 0.05$

Table I

The analgesic action of morphine with or without simultaneous administration of actinomycin D. The analgesic action is shown as the mean reaction time in seconds ± S.E.M. for a group of animals at 30, 60, 90, or 120 min (Reaction Times 3, 4, 5 and 6) after the injection of morphine (10 mg/kg subcutaneously) or morphine plus actinomycin D (10 mg/kg subcutaneously + 10 µg/kg intraperitoneally). Included are the reaction times of the animals 30 min. and just before injection (Reaction Times 1 and 2)

Groups	Days	Reaction Time 1	Reaction Time 2	Reaction Time 3	Reaction Time 4	Reaction Time 5	Reaction Time 6
I[a]	1st	5.7 ± 0.62	4.9 ± 0.74	8.5 ± 0.96	9.4 ± 1.2*	9.7 ± 2.4	5.7 ± 1.2
	2nd	4.2 ± 0.47	5.1 ± 0.60	6.5 ± 1.1	6.6 ± 0.92	4.6 ± 0.55	5.4 ± 0.90
	3rd	4.4 ± 0.69	4.8 ± 0.43	6.5 ± 1.1	6.9 ± 0.91	5.1 ± 0.40	
	4th	4.3 ± 0.65	5.5 ± 0.40	6.1 ± 0.71	4.8 ± 0.48	4.0 ± 0.32	
II[b]	4th	5.7 ± 0.54	6.3 ± 0.48	7.5 ± 0.98	6.1 ± 0.60	4.4 ± 0.42	
III[c]	1st	5.8 ± 0.57	5.6 ± 0.52	9.4 ± 1.6	17.6 ± 4.0*	14.8 ± 4.1	9.8 ± 2.6
	2nd	4.9 ± 0.97	4.9 ± 0.56	7.3 ± 0.60	6.3 ± 1.5	7.5 ± 1.2	
	3rd	5.5 ± 0.73	4.6 ± 0.40	6.3 ± 1.3	6.7 ± 0.85	5.2 ± 0.80	

a) Group I (n = 10) Animals given morphine plus actinomycin D daily for 4 days and tested on the hot plate each day

b) Group II (n = 10) Same treatment as in Group I except that the animals were tested on the hot plate on the 4th day only

c) Group III (n = 6) Animals given morphine daily for 3 days and tested on the hot plate each day

* Mean values significantly different ($P < 0.05$)

Table 2

The analgesic action of morphine (10 mg/kg subcutaneously) in two groups of rats (see table 1)

Groups	Days	Reaction Time 1	Reaction Time 2	Reaction Time 3	Reaction Time 4	Reaction Time 5	Reaction Time 6
IV[a]	1st	5 6 ± 0 29	4 8 ± 0 27	14 7 ± 3 6	17 5 ± 3 6	18 9 ± 3 8	14.2 ± 3 5
	2nd	5 7 ± 0 88	4 7 ± 0 52	10 1 ± 2.5	14 2 ± 3 2	9 7 ± 3 1	4 7 ± 0 49
	3rd	6 0 ± 0 86	4 7 ± 0 42	6 4 ± 0 86	6 6 ± 0 91	6 6 ± 1 1	
	4th	6 3 ± 0 65	4 8 ± 0 53	5 4 ± 0 58*	4 7 ± 0 78	5 0 ± 0 65	
V[b]	4th	5 2 ± 0 51	4.3 ± 0.53	8 1 ± 0 90*	6 9 ± 0 96	5 8 ± 1 0	

a) Group IV (n = 10) Animals given morphine daily for 4 days and tested by the hot plate each day

b) Group V (n = 8) Same treatment as in Group IV except that the animals were tested on the hot plate on the 4th day *only*

* Mean values significantly different ($P < 0\ 05$)

Results

Tolerance to the analgesic action developed rapidly whether morphine was given with or without actinomycin D (table 1, Groups I and III) Simultaneous injections of morphine and actinomycin D, moreover, resulted in a lower degree of analgesia on the first day after 30–120 min than when morphine was given alone (Reaction Times 3, 4, 5, 6, first day) At the height of analgesia the difference was statistically significant (Reaction Time 4, first day) On the fourth day of treatment, the reaction times of the animals in Group II (table 1, animals not tested by the hot plate method until on that day) were only slightly greater than those of the rats in Group I on the same day These differences were not statistically significant Thus tolerance apparently developed to the same degree whether or not the animals had been tested previously The high degree of tolerance to the analgesic action of morphine seen in animals in Group II therefore prompted us to perform two further studies, the results of these experiments are shown in table 2

The reaction times 3, 4, and 5 of rats in Group IV were somewhat lower than those of rats in Group V on the fourth day of treatment The highest value in Group V (Reaction Time 3) was significantly different from the corresponding value in Group IV (table 2)

Rats in Group IV (table 2) showed a higher degree of analgesia (Reaction Times 3, 4, 5, 6) than rats in Group III (table 1) on the first day These differences were not statistically significant However, tolerance appeared to develop more rapidly in the rats in Group III than in Group IV

Discussion and Conclusions

The results presented here do not support the earlier findings that actinomycin D delays or prevents the development of tolerance to morphine analgesia in rats (COHEN *et al* 1965, COX *et al* 1968) On the contrary, it was found that actinomycin D decreases the degree of morphine analgesia in the rats (table 1) This was a rather unexpected finding since LOH *et al* (1971) found that actinomycin D can facilitate access of morphine to the brain thus resulting in higher amounts of morphine in the brain of rats and mice However, it should be noted that these investigators used 50 fold higher doses than those which were used in the present study

The results of the analgesimetric experiments (tables 1 and 2) are in close agreement with the earlier results of JÓHANNESSON *et al* (1965) that a considerable or even a high degree of tolerance is present after only one analgesic dose of morphine The results are, however, somewhat at variance with the findings of KAYAN *et al* (1969) KAYAN & MITCHELL (1969) and

GEBHART & MITCHELL (1971) that two types of tolerance to morphine analgesia exist, i e, tolerance which depends on the interaction between morphine and the test procedure and tolerance which depends on the interaction between morphine and cells in the central nervous system The reaction times 3, 4, and 5 were definitely higher on the fourth day in rats which had not been previously tested than in those which had been tested previously (table 2) However, these values were of a low order of magnitude and the differences between them only became significant at 30 min after the injections (table 2, Reaction Time 3) It should be mentioned here that we used rats of a different stock from those of the above mentioned investigators Inborn differences in rats of various stocks might thus be of importance in this connection

The conclusions of this work are therefore that actinomycin D does not hinder the development of tolerance to morphine analgesia Furthermore tolerance to this action of morphine does not necessarily involve a high degree of behavioural tolerance to the apparatus used for analgesimetric testing

REFERENCES

Cohen M A S Keats W Krivoy & G Ungar Effect of actinomycin D on morphine tolerance *Proc Soc Exp Biol Med* 1965 **119**, 381–384

Cox B M M Ginsburg & O H Osman Acute tolerance to narcotic analgesic drugs in rats *Brit J Pharmacol* 1968 **33** 245–256

Cox B M & O H Osman Inhibition of the development of tolerance to morphine in rats by drugs which inhibit ribonucleic acid or protein synthesis *Brit J Pharmacol* 1970 **38** 157–170

Gebhart G F & C L Mitchell Further studies on the development of tolerance to the analgesic effect of morphine The role played by the cylinder in the hot plate testing procedure *Arch int Pharmacodyn* 1971, **191** 96–103

Goldstein A *Biostatistics An introductory text* The Macmillan Company, New York 1964 pp 51–55

Jóhannesson T & L A Woods Analgesic action and brain and plasma levels of morphine and codeine in morphine tolerant codeine tolerant and nontolerant rats *Acta pharmacol et toxicol* 1964 **21** 381–396

Jóhannesson T L A Rogers J R Fouts & L A Woods Tolerance to morphine and codeine analgesia and hepatic enzymic microsomal drug metabolism in the rat *Acta pharmacol et toxicol* 1965 **22** 255 269

Jóhannesson T W J Steele & B A Becker Infusion of morphine in maternal rats at near term Maternal and fetal distribution and effects on analgesia brain DNA RNA and protein *Acta pharmacol et toxicol* 1972 **31** 353–368

Kayan S & C L Mitchell Further studies on the development of tolerance to the analgesic effect of morphine *Arch int Pharmacodyn* 1969, **182** 287–294

Kayan S L A Woods & C L Mitchell Experience as a factor in the development of tolerance to the analgesic effect of morphine *Eur J Pharmacol* 1969 **6** 333–339

Loh H H, F H Shen & E L Way Effect of dactinomycin on the acute toxicity and brain uptake of morphine *J Pharmacol* 1971, **177** 326–331

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Identification Tests for Bases Formed During the Putrefaction of Visceral Material

By

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Abstract Data has been compiled on the general properties of 29 basic compounds which have been reported to occur as putrefaction products of human viscera. The properties include responses to colour tests, behaviour in selected systems of gas, paper, and thin layer chromatography, and absorbance in the ultraviolet and infrared range The relative efficiencies of some acid protein precipitating reagents have been tested with respect to the release of endogenous bases from liver tissues From this information together with the development of procedures for extracting the bases from chromatograms a number of naturally occurring basic compounds have been isolated from human liver tissues and identified

Key words Post mortem changes – autolysis – chemistry analytical – liver

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The interference of compounds of putrefactive origin in the analysis of viscera for drugs and poisons is a well known problem stemming from the beginning of forensic toxicology 150 years ago

Some putrefactive bases possess maxima in the "working" region of the ultraviolet spectrum, and give positive reactions to certain colour tests which are used to detect drugs, but there has been a general tendency to exclude these compounds from text books on toxicology with the exception of the two simple water soluble diamines, cadaverine and putrescine

There have been some limited applications of alkaloid colour reagents to bases which have been produced in tissues by endogenous means (TRUHAUT & LE MOAN 1958, CLARKE 1962), and at this Establishment a number of putrefaction bases have been included in comprehensive retrieval systems for drugs, based on spectrophotometric properties and behaviour in a thin layer chromatography system (CURRY *et al* 1970)

Information has also been provided on the spectrophotometry, paper chromatography, polarography and partition coefficients of some putre

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factive compounds of acidic, neutral, and basic nature (KÆMPE 1969) Some indications of the interferences which can be caused by basic compounds, formed by putrefaction, in the spectrophotometric and polarographic analysis of alkaloidal drugs have been made, and the suggestion has been advanced that the interference in ultraviolet spectrophotometry can be over come by the addition of putrefactive bases to the reference cell This can not, of course, be done when the maxima of the drug and the putrefactive base coincide Paper chromatography was used by Kæmpe to separate some alkaloid drugs, including morphine, from interfering bases such as tyramine, but his separations in general, required a long period of time to complete Thin layer chromatography was used to separate tryptamine from 2-phenyl ethylamine, but scanty use was made of differential colour reactions or gas chromatographic procedures The latter method has been used to determine putrescine in plant material (SMITH 1970)

As data on putrefactive bases appears to be generally rather scanty and scattered in the literature, it was deemed that it would be of use to forensic toxicologists if an "atlas" of the common properties of these compounds was compiled which included, ultraviolet and infrared spectroscopy, chromatographic data using gas, paper and thin layer methods and differential colour reactions

For this work as many as possible of the bases which have been reported as having been detected in extracts of putrefying visceral material were obtained

Some of these compounds would not be encountered in the usual extracts obtained in British forensic science laboratories, where an aqueous protein free alkaline filtrate is extracted with an organic solvent, which, after washing, is then concentrated by evaporation This is because the compounds are either too water soluble (e g , ethanolamine, putrescine, cadaverine) or too volatile (e g , methylamine, ethylamine) They have been included, however, as some laboratories occasionally use the Stas Otto alcoholic method for extracting viscera Our study also includes nicotinamide and salts of acetylcholine, choline, neurine and histamine Although these bases are not essentially products of protein putrefaction they are present in body tissues

The data in the compiled "atlas" of properties have been applied to characterise endogenous basic material derived from the extraction of *post mortem* liver tissues

Materials and Methods

Sources of the samples

Adenine, harmine piperidine and putrescine (tetramethylene diamine) were obtained from Messrs Koch Light Colnbrook Bucks and samples of harman norharman and δ valerolactam (piperidone) from Messrs Ralph N Emanuel, Wembley Middlesex

Neurine bromide was obtained from Messrs. Kodak, Kirkby, Lancs and nicotinamide from Messrs. Lewis Leeds, Yorkshire N methylnicotinamide was purchased from Messrs Sigma Chemical Co Ltd., London S W 6

A specimen of hydroxy harman was kindly donated by Dr B Kæmpe of the Department of Forensic Medicine, University of Aarhus, Denmark

The remaining bases were purchased from Messrs. British Drug Houses Ltd, Poole, Dorset.

Spectrophotometric analysis

The ultraviolet spectra of the bases were plotted on a Unicam SP800 recording spectrophotometer between the wavelengths of 400 and 200 nm No differences in the wavelengths of the maxima were observed if the spectra were plotted using either 0.5 N or 0 1 N sulphuric acid as the solvent medium The stronger acid (0.5 N) was preferred for the isolation of bases from visceral extracts (see below) to avoid any possibility of neutralisation $E^{1\%}_{1\,cm}$ values of maxima were determined in 0 1 N−H_2SO_4 solution but maxima below 220 nm were not recorded as they are outside the usual working range for drugs

Infrared spectra were obtained on a Perkin Elmer 225 spectrophotometer using the KBr disk method for solids and a sealed cell or smear technique for liquid samples Free amines below butylamine are unlikely to interfere with infrared analyses as total escape of *n* propylamine (B Pt 48°) occurs when starting the plotting of the spectrum of a sample between salt plates

Chromatography systems

a. *Gas*

For this work a Pye Series 104 Gas Chromatograph was employed The column packing was Silicone Gum rubber E301 (SE30) on Chromosorb G AW DMCS 80–100 mesh (21 971) as supplied by Messrs Perkin Elmer Beaconsfield, Bucks and the column consisted of silanized glass 5 ft×⅛ inch. Flow rates were N_2 40 ml/min, H_2 40 ml/min and the method of detection was by flame ionisation The retention times of the more volatile bases were recorded at 100°, and those of the less volatile ones at 200°

Injections were made using 0 1 µl of the more volatile liquid bases, and solutions of the higher boiling solid bases in ethanol

b *Paper*

The Rf values of over 700 alkaloids have recently been described (CURRY *et al* 1970) in a citrated paper system (CURRY & POWELL 1954) which was modified (CLARKE & HAWKINS 1963) The modified system was used for the putrefactive bases

Whatman No 1 paper sheets were soaked in a 5 % w/v solution of sodium di hydrogen citrate and the excess solution removed by blotting paper The sheets were then hung up to dry at room temperature The mobile phase was prepared by dissolving 4 8 g of citric acid in 130 ml of water, and adding 870 ml of n butanol After shaking, the mixture was homogenous

This system, which has proved invaluable for separating basic drugs, gave good separations between some putrefaction bases but there was a tendency for "bunching" to occur in the region of Rf 0–0 3, so that a complementary thin layer system was devised (see below)

c *Thin layer*

A system which gave generally satisfactory separations of putrefactive bases consisted of Merck pre coated aluminium oxide plates F254, type E (obtained from Messrs Anderman and Co, London, SE 1) and a mobile phase which was the upper layer of a mixture prepared by shaking 100 ml of methyl acetate with 50 ml of a mixture of 880 ammonia and water (2 5 97 5 by vol) To obtain the best resolution with this system the room temperature should not exceed 70° F (21° C) Other solvent mixtures were developed for specific separations but these were not suitable when applied to the whole range of the bases Thus ethyl acetate chloroform formic acid 9 9 2, and ethyl acetate chloroform acetic acid 4 14 2, separated tryptamine from tyramine, while water saturated methyl acetate was used to separate dl 1 – from 2 phenylethylamine

Solutions of the bases in methanol, approximately 10 % w/v in strength, were prepared for paper and thin layer chromatography, and diluted as required to avoid overloading Adenine thymine and uracil were used as saturated methanolic solutions

Colour tests

These were carried out either as spot tests, where a few fragments of a solid or a smear of a liquid was placed on a white spot tile and a drop of reagent added, or as plate tests in which the thin layer plate or paper chromatogram was sprayed with the reagent solution in order to locate the positions of the bases after the run in the mobile phase

The plate tests were applied to the bases after chromatography in both the thin layer and paper systems, as it was sometimes found that a given substance would behave differently to the same reagent on the two different types of chromatogram The following is a list of reagents used for the identification tests, with details of their preparation and application

Reagents and methods for spot tests

1 *Concentrated sulphuric acid*
(Sp gr = 1 84)

2 *Marquis reagent*
0 5 ml of 40 % formaldehyde was placed in a stoppered tube, and 10 ml of concentrated sulphuric acid was added The reagent was freshly prepared each day

3 *Liebermann reagent*
Concentrated nitric acid was allowed to drip onto a quantity of copper turnings in a flask fitted with an anti splash device, and the resulting fumes of brown nitrogen peroxide were absorbed into cold concentrated sulphuric acid This reagent was originally designed for p-unsubstituted phenols but was subsequently found to give good colours with various other types of compounds (STEVENS 1967)

4 *Chromic acid*
0 5 g of potassium dichromate was dissolved in 40 ml of water, and 60 ml of concentrated sulphuric acid added to the solution with cooling

5 *Vitali's test*
A drop of fuming nitric acid was placed on the compound, the mixture then being evaporated to dryness on a water bath A drop of ethanolic potassium hydroxide (5 % w/v) was added to the dry residue Any colour changes (excluding yellow and orange to brown, unless very bright) were noted on the addition of acid, in the dry residue and on the addition of alcoholic potash

6. *Frohde reagent*

1 g of ammonium molybdate was dissolved with warming in 100 ml of concentrated sulphuric acid

7. *Mandelin reagent*

1 g of ammonium vanadate was dissolved in 100 ml of warm concentrated sulphuric acid

8. *Mecke reagent*

1 g of selenious acid was dissolved in 100 ml of warm concentrated sulphuric acid

Reagents 2, 5 6 7, and 8 are used in the alkaloid drug identification scheme of CLARKE (1962)

9. *Cyanogen bromide – aniline*

(a) To 50 ml of water saturated with bromine, solid potassium cyanide was added until the solution was just decolorized Bromine saturated water was then added drop by drop to the solution until a pale yellow colour was produced. This solution was then shaken with 50 ml ether, and the ether extract dried with anhydrous sodium sulphate

(b) 50 ml of a saturated solution of aniline in water was shaken with 50 ml ether, and the ether extract dried with anhydrous sodium sulphate

For the test 2 volumes of (a) were mixed with 1 volume of (b)

This reagent gives bright orange red colours with certain compounds containing the pyridine nucleus such as nicotinamide and nicotine and of course pyridine itself If stored separately the ether solutions remain stable for a period of weeks.

10. *Sodium hypochlorite*

The solution was used as supplied by Messrs Fisons Ltd., Loughborough, Leicestershire containing 12 % available chlorine

11. *Simon test*

In an aqueous medium this is a test for aliphatic and heterocyclic secondary amines The reagent was prepared by dissolving 1 0 g of sodium nitroprusside in 50 ml of water and adding 2 ml of acetaldehyde Secondary amines such as pyrrolidine and piperidine give a bright blue colour when added to this reagent in a tube

The "wet" test was done on a spot tile by adding a drop of the reagent to a drop of the sample of putrefactive base under test

The "dry" Simon test is a modification of the original one A strip of Whatman No 1 paper was soaked with a 2 % w/v aqueous solution of sodium nitroprusside and dried in warm air One spot of the solution of the base in methanol was placed on the treated paper, which was then exposed to acetaldehyde vapour

A considerable proportion of the putrefactive bases gave a blue or mauve colour with this test and the production of colour was not restricted to secondary amines

12. *Nessler's reagent*

To a saturated aqueous solution of mercuric chloride solid potassium iodide was added until the red precipitate of mercuric iodide had redissolved An equal volume of 30 % w/v sodium hydroxide was then added to the solution

Reagents and methods for plate tests

13. *UV light*

Radiation of 254 nm was used from a Camag" universal lamp The presence of any fluorescent or absorbing spot was noted and the spot ringed with pencil before the application of any of the colour producing sprays

14 *Iodoplatinate*

5 ml of 5 % chloroplatinic acid solution was diluted with water to 200 ml, and 30 g of potassium iodide and 10 ml of 2 N hydrochloric acid were added to the solution The hydrochloric acid is of value in the spraying of alkaline TLC plates especially if strong bases are present

15 *Permanganate*

2 g of potassium permanganate was dissolved in 100 ml of water

16 *Sulphanilic acid*

Sulphanilic acid (0 9 g) was added to a mixture of water (90 ml) and concentrated hydrochloric acid (9 ml) and the mixture warmed to bring about a solution. After cooling an equal volume of 5 % w/v aqueous sodium nitrite was added to diazotise the sulphanilic acid

A solution of 10 % w/v aqueous sodium carbonate was also prepared For the test the diazotized sulphanilic acid solution was first sprayed lightly onto the plate or paper, followed by a more vigorous application of the sodium carbonate spray

17 *Ninhydrin*

An aerosol container of 'Ninspray" was used for this test This can be obtained from Messrs British Drug Houses, Ltd , Poole Any colours appearing immediately after spraying were noted, and the plate or paper was then heated to 100–110° in a current of hot air and any further appearance of coloured spots recorded

This test yielded good results with volatile amines like methylamine, ethylamine and propylamine on citrated paper at 100–110° It was noted that the test for these compounds was positive after the paper chromatogram had been left on the labora tory bench for several days presumably because the amines are not volatile from the acid buffer on the paper

18 *Fast blue B salt*

This compound was purchased as "Echtblausalz B' from Messrs Anderman and Co , London S E 1 For the test spray approximately 0 5 g was dissolved in 25 ml of water with a few drops of dilute sodium hydroxide to assist solution After spraying the plate or paper with this mixture, a light overspray of dilute sodium hydroxide was then applied

19 *Xanthydrol*

The reagent consists of a 1 % solution of Xanthydrol in 95 % ethanol containing a few drops of concentrated hydrochloric acid After spraying, the plate or paper was warmed in a current of hot air

20 *Ehrlich test*

2 g of *p* dimethylaminobenzaldehyde were dissolved in a warm mixture of 80 ml ethanol and 20 ml of 6 N hydrochloric acid After spraying this solution on to the plate or paper, the chromatogram was heated to 100–110° in hot air

21 *Simon test*

The paper or plate was sprayed lightly with a 2 % w/v aqueous solution of sodium nitroprusside A 10 % solution of acetyldehyde in methanol was then sprayed onto the chromatogram and the appearance of colours noted

A modification of this plate test was to spray a blank thin layer alumina plate with 2 % w/v aqueous sodium nitroprusside and dry it in warm air Spots of the mixed solution of bases in methanol were applied, and after running in the mobile phase the plate was exposed to acetaldehyde vapour Blue colours resulted from a number of compounds not all of which were secondary amines The modified Simon test would enable dry nitroprusside treated plates to be prepared and stored ready for use and these required no spraying to develop them after elution

22 *Oxidising mixture*

To 49 ml of approximately 30 % w/v sulphuric acid 1 ml of 5 % w/v aqueous ferric chloride was added This mixture was added to an equal volume of 36 % w/v perchloric acid After spraying, the chromatograms were heated to 100–110° in a current of hot air.

23 *Citric-anhydride*

This was prepared by dissolving 1 g of citric acid powder in 50 ml acetic anhydride Chromatograms were heated to 100–110° after spraying with this solution Reagents (16), (19), (20), (22) and (23) were used by McIsaac *et al* (1967) to detect β-carbolines

24 *Tetrazotised tolidine*

This reagent has been used as a reagent for cannabinols (Stone & Stevens 1969) on account of its colour reactions with phenolic compounds in general 5 g of o-tolidine was mixed with about 200 ml of water and 25 ml of 25 % hydrochloric acid. After the solid material had dissolved by warming the mixture, the latter was diluted to 1 litre Equal volumes of this solution and a 10 % w/v aqueous solution of sodium nitrite were mixed together and used as the detecting spray

25 *Cobalt thiocyanate*

3 g of cobalt nitrate was dissolved in 50 ml of water, to which 9 g of potassium thiocyanate was added The thin layer plate or paper chromatogram was lightly sprayed with this mixture and any coloured spots appearing at room temperature were recorded The chromatograms were then heated to 110° and any further coloured spots noted This reagent has been used for detecting certain basic drugs on chromatograms (Curry 1959)

26 *Bromocresol green*

A solution of bromocresol green (pH 4 0 to 5 4) as supplied by Messrs Hopkin and Williams Ltd, was sprayed onto the chromatogram

27 *Citric acid – bromocresol green*

This was applied to the thin layer plate only, as the results of Test 26 on this type of chromatogram were inferior to those obtained on citrated paper As the alumina plate was alkaline after running it in the eluant, it was sprayed with 5 % w/v aqueous citric acid before the application of the bromocresol green spray The plate was finally exposed to ammonia vapour Bromocresol green was used by Clarke (1962) to identify alkaloid drugs on citrate buffered paper sheets

28 *Ammoniacal silver nitrate*

This was prepared by dissolving 1 g of silver nitrate in 20–30 ml of water and adding ammonia solution until the initial precipitate re dissolved The solution was made up to 100 ml with water After spraying, the chromatograms were warmed in hot air

29 *Mercurous nitrate*

A saturated solution in water was prepared The alumina plate, probably on account of its alkalinity, gave a better response than the acid citrate paper If the latter was made alkaline a general blackening took place which made the detection of spots very difficult

30 *Folin Ciocalteu*

[illegible]

31 *Copper sulphate – HCl*

To 50 ml of 0 5 [illegible]

concentrated hydrochloric acid was added The chromatograms were heated after spraying them with this mixture

32 *Biuret alkaline copper*

The chromatograms were lightly sprayed with 31, and then with a 10 % w/v aqueous solution of sodium hydroxide

33 *DANSYL chloride (5 dimethylaminonaphthalene sulphonyl chloride)*

Biogenic amines have been reported to give fluorescent 'DANSYL' derivatives (CREVELING *et al* 1968) The reagent solution was prepared by dissolving 0 1 g of DANSYL' chloride in 50 ml of acetone This solution was used for spot tests or plate tests examination being made under UV light (350 nm)

34 *[4 Methyl]o aceto-acetyl phenol*

BAKER *et al* (1952) recorded that primary amines gave brilliantly fluorescent products with *o* acetoacetylphenol For this test the 4 methyl derivative of this compound was dissolved in ethanol to give an approximately 1 % w/v solution which was used for either spot or plate tests

The spot tests with reagents 33 and 34 were best carried out on paper by placing a spot of the methanolic solution of the amine on a piece of Whatman No 1 filter paper and spraying or spotting the paper with the reagent solution followed by examination under ultra violet light (350 nm)

Alternatively a piece of Whatman paper could be soaked with the reagent and dried and the amine solution spotted on to it

35 *Gibb s reagent*

The reagent was reported to give colours with a variety of amines (Ross 1968) For the tests a 1 % w/v solution of 2,6-Dibromoquinone 4-chloroimide was prepared in ethanol

36 *Carbon disulphide*

The TLC plate which must be weakly alkaline or neutral was placed in carbon disulphide vapour for 5 minutes and then viewed in *UV light (254 nm)* Primary and secondary alkyl and heterocyclic amines which usually have poor UV absorption *per se* can be detected by a large increase in UV absorbance due to the formation of dithiocarbamates *in situ* on the plate λ max and $E^{1\%}_{1\,cm}$ values were determined for a number of dithiocarbamate derivatives and these may be useful in helping to identify any particular amine

If sufficient amine is present in the original spot a spray of 1 % silver nitrate in 2 N nitric acid after carbon disulphide treatment will produce dark brown colours (silver sulphide) This colorimetric approach has been used (BASTOS *et al* 1970) to detect amines on a plate but it is not as sensitive as the UV absorbance of the dithiocarbamates

Results and Discussion

The results obtained from gas, paper and thin-layer chromatography, and the responses of the bases to the colour reagents are included in table 1, UV spectra of the six commonly encountered bases are appended

Figs 1 and 2 show the resolution obtained of prepared mixtures of bases by the GLC and TLC methods respectively

Distinguishing between an alkaloid drug and a putrefactive base is not a clearcut matter as there appears to be no diagnostic test for such a distinc-

Table 1.

Atlas of properties of putrefactive bases

Compound	UV Max nm	$E^{1\%}_{1 cm}$	GLC		TLC	Paper	Identification Tests
			Temp °C	Ret Time *Mins Secs*	Rf Value	Rf Value	
Acetylcholine (chloride)			100 200	ni	0 00	0 03	(3) yellow, (4) red, (14) purple, (25) blue, (26) white [TLC], blue [paper], (27) white, (28) blue black [chloride ion], (29) white [TLC], (30) yellow [TLC], (31) yellow [chloride ion], (33) green
Adenine	263	985	200	ni	0 30	0 12	(10) orange, (13) absorbs, (18) red [TLC], (28) white [paper], (29) white [TLC]
iso-Amylamine			100	0 – 38	0 43	0 35	(11) purple, (14) brownish purple [TLC], grey [paper], (16) white [TLC], yellow [paper], (17) purple [TLC, paper at 100°], (18) yellow [TLC], orange [paper], (20) yellow [paper], (21) mauve, (25) blue [paper cold], (26) white [TLC], blue [paper], (27) white, (28) white [TLC], (30) blue [TLC], (33) green f [spot test], yellow f [TLC] green f [paper], (34) yellow green f [spot test], green f [TLC, paper using NH_3], (35) grey-purple [TLC], purple [paper with NH_3], (36) absorbs, λ max = 253, 284 nm, $E^{1\%}_{1 cm}$ = 800, 850
iso Butylamine			100	0 – 29	0 43	0.20	(4) brown, (11) mauve, (14) brown [TLC], (16) white [TLC], yellow [paper] (17) purple [at 100°], (18) orange, (20) yellow [paper], (21) mauve, (25) blue [paper, cold], (26) blue [paper], (27) white, (28) white [paper], (33) green f [spot test], yellow f [TLC], green f [paper], (34) yellowish green f [spot test], green f [paper with NH_3], (35) grey-purple [TLC], purple [paper with NH_3] (36) absorbs λ max = 254, 283 nm $E^{1\%}_{1 cm}$ = 900 1000

Table 1.
(continued)

Compound	UV Max nm	$E^{1\%}_{1\,cm}$	GLC		TLC	Paper	Identification Tests
			Temp °C	Ret Time Mins Secs	Rf Value	Rf Value	
Cadaverine (Pentamethylene diamine)			100	2 – 5	0 00	0 00	(11) mauve, (14) brown [TLC], grey [paper], *(16)* white [TLC], yellow [paper], (17) brownish purple [TLC, 100°], purple [paper, 100°], (18) yellow [TLC], *orange [paper], (20) yellow [paper], (21) mauve,* (23) brown [paper], (24) yellow [TLC], (26) white [TLC], blue [paper], (27) white [TLC with NH_3], (30) blue, (31) yellow [paper], (33) green f [spot test], yellow f [TLC], green f [paper], (34) yellowish green f [spot test], green f [paper with NH_3], (35) brown [TLC], purple [paper with NH_3], (36) absorbs, λ max = 252, 283 nm, $E^{1\%}_{1\,cm}$ = 1050, 1050
Choline (chloride)			100 200	ni	0 00	0 03	(3) yellow, (4) blood red, (14) purple [TLC], grey [paper], *(25) blue [TLC, 100°], blue [paper, cold],* (26) white [TLC], blue [paper with NH_3], (27) white [TLC with NH_3], (28) blue-black [chloride ion reacting], (29) white [TLC], (30) yellow [TLC], (31) yellow [chloride ion reacting], (33) green f, (34) blue f [spot test], absorbs [paper]
Ethanolamine			100	0 – 37	0 05	0 10	(11) purple [wet], blue [dry], (14) white *[TLC], (16)* white [TLC], yellow [paper], (17) brown [TLC, 100°], purple [paper, 100°], (18) orange, (20) yellow [paper], (21) mauve, (23) brown [paper], (24) yellow [TLC], orange [paper], (25) yellow [TLC, 100°], yellow [paper, cold], (26) white [TLC], blue [paper], (27) white [TLC with NH_3], (28) white [paper], (30) blue [TLC], (31) blue [TLC], yellow [paper], (32) violet blue, (33) yellow f [spot test, TLC], green f [paper], (34) yellowish green f [spot test] green f [TLC, and paper with NH_3], (35) greyish brown [TLC], purple [paper], (36) absorbs λ max = 252 284 $E^{1\%}_{1\,cm}$ = 800 900

Table I.
(continued)

Compound	UV Max nm	$E^{1\%}_{1\,cm}$	GLC		TLC	Paper	Identification Tests
			Temp °C	Ret. Time Mins Secs.	Rf Value	Rf Value	
Ethylamine			100	0 – 24	0 03	0 10	(11) mauve [dry], (16) white [TLC], yellow [paper], (17) bluish-purple [TLC and paper at 100°], (18) orange, (20) yellow [paper], (21) mauve, (26) blue [paper], (28) white [paper], (31) yellow [paper], (33) green f [spot test], yellow f [TLC], (34) yellowish green f [spot test], green f [paper with NH_3], (35) purple [TLC, paper with NH_3], (36) absorbs.
Harman	247 300	2000 1100	200	4 – 15	0 96	0 50	(1) pink, (2) green (3) dark brown, (4) greenish grey, (5) Red/red/red, (6) blue to green, (7) green, (8) reddish purple, (12) grey, (13) dark blue f [TLC], blue f [paper], (14) yellowish brown [TLC], grey [paper], (18) orange [TLC], yellow [paper], (22) mauve [paper], (24) red [TLC], (25) blue [TLC cold and 100°, paper cold], (26) white [paper], (27) white [TLC with NH_3], (28) yellow [TLC], white [paper], (30) blue, (33) blue f, (34) blue f [spot test, paper], dark blue f [TLC], (35) grey [TLC] [slow reaction]
Harmine	247 319 366	1942 988 303	200	10 – 30	0 94	0 40	(1) greenish yellow, (2) reddish orange, (3) dark brown to violet brown, (4) dark brown to violet brown (5) green/grey/orange, (6) yellow to grey, (7) blue to green, (8) green, (13) dark blue f [TLC], blue f [paper], (14) yellowish brown [TLC], purple [paper], (17) pink [paper, 100°], (18) orange [TLC], (22) mauve [paper], (24) red [TLC], (25) blue [TLC], (26) white [paper], (27) white [TLC with NH_3], (30) blue, (33) blue f, (34) blue f [spot test, paper], dark blue f [TLC], (35) grey [TLC] [slow reaction]

Table 1.
(continued)

Compound	UV Max nm	$E^{1\%}_{1\,cm}$	GLC		TLC	Paper	Identification Tests
			Temp °C	Ret Time Mins Secs	Rf Value	Rf Value	
Histamine (chloride)			100 200	ni	0 25	0 00	(3) yellow, (4) red, (10) pink to orange, *(14) brown* [TLC], (16) red, (17) brown [TLC, 100°], greyish blue [paper, 100°], (18) reddish brown [TLC], orange-red [paper], *(24) grey [TLC]* [slow reaction], (25) yellow [TLC, 100°], (28) blue-black [chloride ion reacting], (29) white [TLC], (30) blue, (32) violet blue [TLC], (33) yellow [TLC], (35) greyish purple [TLC], purple [paper with NH_3]
Hydroxyharman	247 300	*1190* 660	200	*ni* (25 min)	0 50	0 50	(2) green, (3) dark brown, (4) greyish blue, (5) red/red/red, (6) blue to green, *(7)* blue to green, *(8)* yellow, (13) dark blue f [TLC], blue f [paper], (14) purple, (15) decolorized,, (18) orange [TLC], (24) *orange [TLC], (33) blue f,* (34) blue f.
Methylamine			100	0 – 24	0 03	0 00	(11) mauve [dry], (16) white [TLC], yellow [paper], (17) reddish purple [TLC and paper at 100°], (18) orange, (20) yellow [paper], (21) mauve, (28) white [paper], (33) yellow f [spot tests and TLC], green f [paper], (34) yellowish green f [spot test], green f [paper with NH_3] (35) greyish purple *[TLC]*, purple [paper with NH_3], (36) absorbs, λ max ≈ 250, 281 nm
Neurine (bromide)			100 200	ni	0 00	0 05	*(6) greyish* green to blue, (14) purple [TLC], grey [paper], (15) decolorized, (16) yellow [TLC], (22) orange [paper] [Br_2 liberated], (25) blue [TLC 100°, paper cold], (26) white, (27) white [TLC with NH_3], (28) blue-black [Br^- ion reacting], *(29)* white *[TLC]*, (30) yellow [TLC], (31) brown [Br^- ion reacting] (33) green f

Table 1
(continued)

Compound	UV Max nm	$E^{1\%}_{1\,cm}$	GLC		TLC	Paper	Identification Tests
			Temp °C	Ret. Time Mins Secs	Rf Value	Rf Value	
Nicotinamide	220 261	520 454	200	0 52	0.70	0.50	(9) orange (13) absorbs (14) yellow (16) yellow [TLC] (21) yellow when dry (25) yellow [TLC 100°] (26) white (27) white [TLC with NH_3] (28) white [paper] (29) white [TLC] (31) blue [TLC] (33) absorbs (34) absorbs [TLC] (35) grey [TLC] [slow reaction]
N Methylnicotinamide	262	410	200	0 59	0.80	0.70	(9) orange (13) absorbs (14) yellow (33) absorbs (34) absorbs [TLC] (35) grey [TLC] [slow reaction]
Norharman	247 300	1726 910	200	4 15	0.96	0.40	(1) yellow (2) green (3) brown to crimson (4) dark brown (5) yellow/yellow orange (6) blue to green (7) blue, (8) violet (13) dark blue f [TLC] blue f [paper] (14) brown [TLC] grey [paper] (17) pink [paper 100°] (18) orange [TLC] yellow [paper] (22) yellow [TLC] mauve [paper] (23) brownish violet [TLC] (24) red [TLC] (25) blue [TLC and paper cold] (26) white [paper] (27) white [TLC with NH_3] (28) white [paper] (30) blue (31) yellow [TLC] brown [paper] (33) blue f (34) blue f (35) grey brown [TLC]
Phenylethylamine	257 252 263	20	100	2 40	0.88	0.35	(1) yellow (2) red brown (3) reddish orange (8) dark brown (11) blue [dry] (13) absorbs (14) brown [TLC] (16) white [TLC] yellow [paper] (17) brown [TLC and paper at 100°] (18) yellow [TLC] orange [paper] (20) yellow when dry [TLC] (21) grey [TLC] brownish grey [paper] (24) orange [TLC] (25) blue [TLC cold and 100° paper cold] (26) blue [paper] (27) white (28) white [paper] (29) white [TLC] (30)

Table 1.
(continued)

Compound	UV Max nm	$E^{1\%}_{1\,cm}$	GLC		TLC	Paper	Identification Tests
			Temp °C	Ret Time Mins Secs	RF Value	Rf Value	
							blue [slow reaction], (31) yellow [paper], (33) green f, (34) yellowish green f [spot test] green f [TLC, paper with NH_3], (35) pinkish orange [TLC], purple [paper with NH_3], (36) absorbs, λ max = 253, 287, $E^{1\%}_{1\,cm}$ = 1400, 1350
2 Phenylethylamine	258 252 263	20	100	3 – 30	0 60	0 30	(2) blood red, (3) blood red, (8) dark brown, (11) mauve [dry], (13) absorbs, (14) brown [TLC], grey [paper], *(16) white [TLC], yellow [paper], (17) purple* [TLC cold], brownish purple [TLC, 100°], blue purple [paper at 100°], (18) yellow, (20) yellow when dry [TLC], (21) mauve, (24) orange [TLC] (25) blue *[TLC cold and 100°, paper cold], (26) blue [paper],* (27) white [TLC], (28) white [paper], (29) grey [TLC], (30) blue [slow reaction], (31) yellow, (33) green f, (34) yellowish green f [spot test], green f [TLC, paper *with NH_3], (35) purple [TLC, paper with NH_3],* (36) absorbs, λ max = 253, 285 nm, $E^{1\%}_{1\,cm}$ = 1100, 1050
Piperidine			*100*	*0 – 47*	*0 30*	*0 10*	*(11) blue [wet and dry] (14) greyish blue, (16) white* [TLC], (17) purple [paper, 100°], (18) orange, (20) yellow when dry [TLC], (21) blue [TLC], mauve [pa per], (24) yellow [TLC], (25) blue [TLC 100°, paper cold], (26) blue [paper], (27) white [TLC with NH_3] (28) white [paper], (30) yellow [TLC], blue [paper], (31) orange brown [TLC], yellow [paper], (33) green f, (34) yellowish green f [spot test], absorbs [TLC] green f [paper with NH_3], (35) pink [TLC], purple [paper with NH_3], (36) absorbs, λ max = 261 279 nm, $E^{1\%}_{1\,cm}$ = *1650, 1600*

Table I
(continued)

Compound	UV Max nm	$E^{1\%}_{1cm}$	GLC		TLC	Paper	Identification Tests
			Temp °C	Ret Time Mins Secs	Rf Value	Rf Value	
Piperidone			200	0–46	0 60	0 70	(16) yellow [TLC], (20) yellow when dry [TLC], (24) yellow [TLC] [slow reaction], (25) blue [TLC 100°, paper cold], (27) white, (30) blue [TLC], (33) green f, (34) green f [TLC], (35) grey [slow reaction]
n Propylamine			100	0–26	0 15	0 13	(11) blue [dry], (16) white, (17) purple [TLC and paper at 100°], (18) orange, (20) yellow [paper], (21) mauve [TLC], (26) blue [paper], (28) white [paper] (31) yellow [paper], (33) green f [spot test] yellow f [TLC], green f [paper], (34) yellowish green f [spot test], green f [paper with NH_3], (35) purple, (36) absorbs.
Putrescine (Tetramethylene diamine)			100	1–13	0 00	0 00	(8) reddish brown, (11) blue [wet], mauve [dry], (14) brown [TLC], (16) white [TLC], yellow [paper], (17) purple [TLC and paper at 100°], (18) orange [TLC], yellow [paper], (20) yellow, (21) mauve [TLC], violet-blue [paper], (24) greyish yellow [TLC], orange [paper], (25) yellow [TLC 100°], white [paper cold], (26) white [TLC], blue [paper], (27) white [TLC with NH_3], (28) white [paper], (29) black to white in 5 mins. [TLC], black [paper] (30) yellow, (31) blue [TLC], yellow [paper] (32) violet blue, (33) green f, (34) yellowish green f [spot test], green f [TLC, paper], (35) brownish purple [TLC], purple [paper] (36) absorbs, λ max = 255, 279 nm
Pyridine	257	1650	100	0–43	0 35	nd	(9) brick red, (27) white [TLC with NH_3], (33) green f [spot test]

Table 1.
(continued)

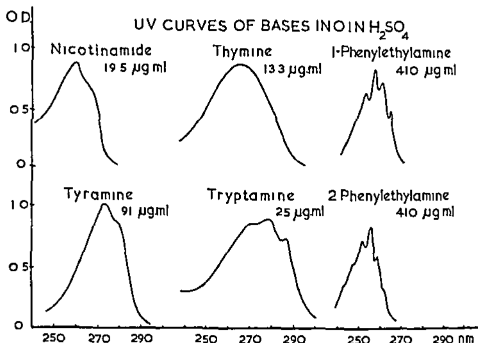
Compound	UV Max nm	$E^{1\%}_{1\,cm}$	GLC		TLC	Paper	Identification Tests
			Temp °C	Ret Time Mins Secs	RF Value	Rf Value	
Pyrrolidine			100	0 – 36	0 05	0 05	(11) blue (14) purple [TLC], grey [paper], (16) white [TLC], (17) yellow [paper at 100°], (18) yellow, (20) yellow [paper], (21) blue, (23) brown [paper], (24) yellow [TLC], orange [paper] [slow reaction], (25) blue [TLC 100°] (26) blue [paper] (27) white [TLC with NH_3] (28) white [paper], (30) blue, (31) orange [TLC] yellow [paper], (33) yellowish green f [spot test], green f [TLC and paper], (34) yellowish green f [spot test], green f [TLC, paper with NH_3], (35) purple [TLC, paper with NH_3], (36) absorbs, λ max = 255, 277 nm, $E^{1\%}_{1\,cm}$ = *1250, 1200*
Thymine	266	640	200	2 – 36	0 32	0 45	(12) grey [slow reaction], (13) absorbs, (15) decolorized [TLC], slow reaction [paper] (29) white [TLC]
Tryptamine	272 278 287	336 349 285	200	2 – 15	0 57	0 20	(1) greenish yellow, (2) yellowish brown, (3) dark brown, (5) orange/orange/orange, (6) green, (8) dark green, (11) blue [wet], mauve [dry], (13) dark blue f, (14) brownish purple [TLC], purple [paper], (15) decolorized, (16) white [TLC], yellow, [paper] (17) purple [TLC and paper at 100°] (18) yellow, (19) magenta [paper], (20) brown [TLC], violet [paper] (21) bluish mauve [TLC], (22) orange [TLC] orange-brown [paper] (24) yellow [slow reaction on paper], (27) white, (28) white [paper], (29) white [TLC] (30) blue, (31) pink [TLC], purple [paper], (33) green f, (34) yellowish green f [spot test], absorbs [TLC, paper] (35) purplish brown [TLC] purple [paper with NH_3], (36) absorbs, λ max = 259, 275, $E^{1\%}_{1\,cm}$ = 600, 650

Table 1.
(continued)

Compound	UV Max nm	$E^{1\%}_{1\,cm}$	GLC Temp °C	GLC Ret Time Mins Secs	TLC RF Value	Paper Rf Value	Identification Tests
Tyramine	222 275	520 110	200	0–58	0 40	0 20	(2) red brown, (3) dark brown, (5) yellow/yellow/orange, (6) blue to yellow, (8) grey, (11) mauve [dry], (13) absorbs, (15) decolorized, (16) red [TLC], orange [paper], (17) purple [TLC and paper at 100°], (18) orange brown [TLC], reddish orange [paper], (20) yellow when dry [TLC], (21) mauve [TLC], (22) mauve [paper], (24) orange [TLC], (27) white [TLC with NH_3], (28) white [paper], (30) blue, (33) dark yellow f [spot test], green f [TLC], dark yellow f [paper], (34) yellow f [spot test], (35) purplish brown [TLC], (36) absorbs, λ max. = 257, 273 nm, $E^{1\%}_{1\,cm}$ = 600, 450
Uracil	260	770	200	ni	0 22	0 27	(12) grey [slow reaction], (13) absorbs, (29) white [TLC]

Notes on table 1 ni = does not issue from GLC column nd = not detected f = fluorescence

With the Identification Tests limitations of the responses are given in parentheses. Thus "(25) blue [TLC 100° paper cold]" means that a blue colour is given with reagent (25) on a TLC plate at 100° and not in cold, and on paper in the cold only



tion *In general* the putrefactive bases tend to give the reactions expected of simple primary and secondary aliphatic amines These contrast considerably with the responses of the more complex tertiary amine structures usually found in alkaloidal drugs Responses to potassium iodoplatinate tend to be less definite and result in greyish or brownish purples or blues The endogenous bases respond to ninhydrin giving bluish purple colours, and also to the nitroprusside acetaldehyde (Simon) reagent yielding intense blue for secondary amines like piperidine or pyrrolidine and purple for primary amines like isoamylamine or ethanolamine None of the bases tested gave a blue or violet colour with Marquis'reagent, so that potential false positive tests for morphine type drugs are eliminated Unlike tertiary amines, the putrefactive bases which contain aliphatic primary or secondary amine groups react with carbon disulphide and yield UV-absorbing dithiocarbamates

As regards movement in the paper and TLC systems there is a tendency for the endogenous bases to be less mobile than many of the basic drugs which are encountered Many of the putrefactive bases bunch together in the Rf value 0-0 3 region in the paper system and, with the exception of 1-phenylethylamine and the harman compounds, reach Rf values from 0 to 0 7 in the TLC system In contrast 24 common basic drugs when run in the TLC system moved near to the solvent front with Rf values between 0 85 and 1 0, with the exception of morphine of Rf value 0 6 The basic drugs examined included

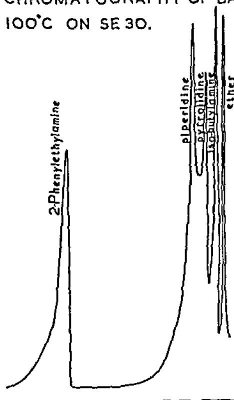


Fig 1 Resolution at 100° on a column of Silicon Gum Rubber E301 Chromosorb G 2½ 97½ w/w of a mixture of A 2 Phenylethylamine, B Piperidine, C. Pyrrolidine, and D Isobutylamine E is the ether solvent peak.

Amitriptyline	Ephedrine	Pheniramine
d-Amphetamine	Imipramine	Phenylpropanolamine
Antazoline	Methaqualone	Propoxyphene
Brompheniramine	Morphine	Protriptyline
Chlorpheniramine	Nicotine	Quinine
Chlorpromazine	Nortriptyline	Strychnine
Codeine	Pethidine	Tranylcypromine
Diphenıramine	Phenelzine	Trifluoperazine

Of the putrefactive bases examined, only harman, *nor*-harman, harmine and 1-phenylethylamine moved to near the liquid front

Thus chromatographically retarded spots giving poor iodoplatinate but good ninhydrin, Simon test, or carbon disulphide responses are an indication of an endogenous base

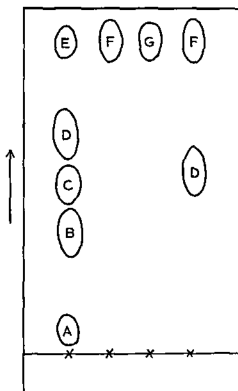


Fig 2 Separation of some amines on a Merck aluminium oxide plate (F 254, Type E), using methyl acetate – 2½ % ammonia A=ethanolamine, B=tyramine or *iso*-amylamine, C=tryptamine, D=2 phenylethylamine, E=1 phenylethylamine, F=amphetamine, G=ephedrine Time of run is approximately 20–30 mins for 10 cm

amine, thymine, nicotinamide and the phenethylamines, possess UV curves of characteristic shape and λ max values.

Analysis of liver samples

It is a common general procedure in British forensic science laboratories to precipitate proteins in a visceral slurry with an acid protein-coagulant, and then to extract the resulting filtrate or centrifugate from this mixture at the appropriate pH, with an organic solvent The extracts obtained are then washed with water and evaporated.

The protein precipitants used here were sodium tungstate-sulphuric acid (VALOV 1946), hot N hydrochloric acid saturated with ammonium sulphate (NICKOLLS 1956), 5 N hydrochloric acid at 70–80° (DUBOST & PASCAL 1953 & 1955) and 10 % aluminium chloride in 2 N hydrochloric acid at 40–50° (STEVENS 1967).

A number of livers, which had been found to be free of basic drugs, were obtained and 50 gram samples of each liver were taken for analysis using

each of the 4 protein precipitation methods The acid filtrates, after a "clean up" with ether, were made alkaline to pH 9 5–10 with ammonia and extracted with an equal volume of ether and then with a quarter of the volume of chloroform

These extracts were dried with a little anhydrous Na_2SO_4, without washing them with water, and evaporated carefully to a few drops The two concentrated extracts were then spotted side by side onto a citrate buffered paper sheet, leaving one third of the paper blank for preparing blank extracts for the reference cell of the ultraviolet spectrophotometer (see below)

The paper was then run in the butanol-citric acid solvent system for up to 5 hours, and, after drying, was examined in ultraviolet light (254 nm) Any absorbing or fluorescing bands were noted and a drawing or "Xerox" copy made for reference purposes

The paper was then divided up giving ten zones each for the portions of the paper sheet bearing the ether and chloroform extracts and also ten "blank" zones, each series of ten representing Rf values of 0–0 9 inclusive

Each of the zones was then cut out and extracted with 1 ml of 0 5 N-H_2SO_4 at 40–50° for 5 minutes The acid extracts so obtained were scanned in turn against the corresponding "blank" extract in the micro-cells of the ultraviolet spectrophotometer from 400 to 200 nm This extraction method had previously been found to give consistently good recoveries of alkaloidal drugs from paper, and was more reliable than the direct chloroform extraction of alkaline paper (CLARKE 1962) The method was also used effectively to extract endogenous bases from the alumina removed from thin layer plates

A record was kept of the method of protein precipitation, the extraction solvent used, the Rf values of the bands excised and the optical density and wavelength of the maxima obtained, and from the results obtained, a number of general observations were made as follows

1 The protein precipitating techniques used could be placed in an order of diminishing efficiency of release of the putrefactive bases from liver tissues as follows
   Ammonium sulphate > aluminium chloride > tungstic acid > Dubost Pascal which alters the entire pattern (see fig 3)
   Ammonium sulphate was the most efficient of the methods tried, and care was taken in this method to keep the acid (HCl) concentration during protein precipitation at least N in strength which is necessary in the authors' opinions to prevent dissociation of weaker bases resulting in poor recoveries
2 Solvent extractable endogenous basic compounds were in general totally extracted by ether from the alkaline filtrates and only occasionally did a subsequent extraction with chloroform yield any additional

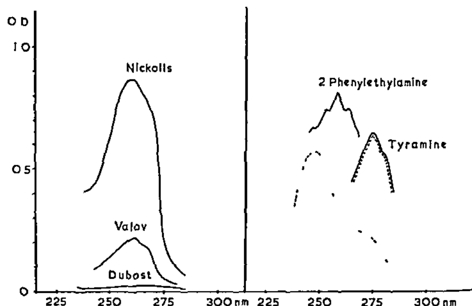


Fig 3 a Example showing the relative amounts of endogenous base (nicotinamide) released from liver tissue using the NICKOLLS (1956) (ammonium sulphate) VALOV (1954) (tungstic acid) and DUBOST PASCAL (1953) (hot hydrochloric acid) methods for protein precipitation b Comparison of the effects of the DUBOST PASCAL and NICKOLLS methods on the recovery of 2 phenylethylamine and tyramine from putrefied liver tissue UV spectra plotted ——— for NICKOLLS and – – – – for DUBOST PASCAL method Tyramine is stable to both methods but 2 phenylethylamine is broken down by the DUBOST PASCAL method

This can be used to advantage if a chloroform soluble basic drug is being sought in visceral material containing putrefaction bases, as a preliminary ether extraction would give a "clean up" procedure

3 Almost all UV absorbance is confined to the range 235 to 275 nm with regions of maximal activity around 260 and 275 nm The amount of absorbance increases rapidly with increase of putrefactive decomposition and this emphasises KÆMPE'S observation (1969) that storage of viscera at −18° is necessary if putrefactive interference in analysis is to be avoided

4 The intensity of fluorescence or absorbance seen in the bands on the paper in UV light usually bears little relation to the amount of UV absorbance of the acid extracts of those bands It would seem that highly fluorescent compounds can be detected visually in trace amounts on the surface of the paper, but when put into a relatively large volume of liquid in the spectrophotometer microcell the concentration is too low to permit a reasonable response to be given by the instrument

The presence of an absorbing substance may be falsely indicated on account of the contrast provided by a strip of empty paper sandwiched between two fluorescent bands Extraction of the dark band and UV assay of the extract is, therefore, advisable for detecting any UV absorbing compound in it

Identification of extracted compounds

Initial evidence was provided by the λ max values and shape of the UV curves obtained on the 0 5 N sulphuric acid extracts of the paper strips taken from the chromatogram, and the Rf values of these strips

The bases were re-extracted from these solutions by ether under alkaline conditions and the ether solutions so obtained were concentrated and run on alumina plates, together with control spots of the bases suspected, in the thin layer system described above



Fig 4 Infra red spectra obtained from material eluted from a TLC plate (plotted) and from a control sample of thymine (plotted ———) An expanded scale was on an external recorder attached to a Unicam SP200 spectrophotometer

Table 2

Identification of endogenous basic compounds obtained from liver tissue samples in various states of putrefaction Isolation of the compounds from the liver samples was carried out using the ammonium sulphate method for protein precipitation (NICKOLLS 1956) with ether extraction of the filtrate at pH 10

1 *Source of Material*	2 *Rf Value of paper strip extracted with* $0.5\,N-H_2SO_4$	3 *UV Max of extract (nm)*	4 *TLC of re extracted base*	5 *Infra red spectrum*	6 *Compound Identified*	7 *Amounts present in extract from 50 g liver calc by* $E^{1\%}_{1\,cm}$ *value*
Fresh liver from young male road accident victim	0 5	261	Band at Rf 0 7 giving orange red colour with re agent (9) Coincident with nicotinamide but not with N methyl nicotinamide* (Rf 0 8)	Mainly nicotinamide	Nicotinamide	200 μg
Liver from 47 year old female found dead in bed Liver stored 1 week at 4° Fresh smell, no putrefactive odour	0 3	275	Band at Rf 0 6 gives red colour with reagent (16) coincident with tyramine control	Mainly tyramine	Tyramine	500 μg
Liver from man found drowned in a ditch No drugs found, slight putrefactive odour	0 2 (Tryptamine= 0 2 Ethyltrypt amine=0 5)	272 278 287	Not tried	Retrieved as tryptamine or ethyltrypt amine (cf column 2)	Tryptamine	1120 μg

Liver of male dying from bronchial pneumonia No basic drugs present Stored at 4° for 2 months mould spots present on liver	0 5	265	Band at Rf 0 25 coincident with thymine or adenine but not with uracil	Thymine	Thymine†	125 μg
Liver deliberately putrefied in an enclosed vessel at 22° *for 14 days in the open air*	0 3	257 252 263	*Band at Rf 0 5* coincident with *2 phenylethylamine*	*Poor spectrum* probably 2-*phenylethyl* amine	*2 phenylethyl*-amine	*820 μg*
Very strong putrefactive odour, maggots present sample semi liquid	0 4	257 252 263	Band at Rf 0 8 coincident with 1 phenylethylamine	Good spectrum of 1 phenylethyl amine	1 phenylethyl-amine	500 μg

* *Reported to occur in human urine* (WARREN & SCOTT 1969)

† Although thymine is not a base, its very weak acid properties coupled with its sparing solubility in organic solvents causes a considerable proportion of it to escape into the basic fraction

When dry, each plate was examined in UV light (254 nm) and after shielding the main portion of the band bearing the substance for identification, a selected colour spray was applied to the unprotected part of the plate including the control spots The Rf value of any reacting band and its position relative to the control spots was noted The major portion of the band on the protected part of the plate was removed and re extracted into warm 0 5 N sulphuric acid The UV curve of this extract was then compared with the original curve obtained from the paper extract as a check

This procedure provided additional information towards the identity of the material under examination It also cleaned up the base from any co-extracted impurities which were not removed by the paper system and improved the quality of the infrared spectrum obtained on the material (see below)

From the acid extract of the thin layer plate the cleaned up base was again recovered by ether extraction under alkaline conditions and submitted to infrared spectrophotometry using a micro-disc technique (CURRY *et al* 1968), for final identification (see fig 4)

In table 2 some typical examples are given where endogenous bases have been identified in samples of liver tissue in various states of putrefaction

From results so far obtained it appears that nicotinamide (λ max 261 nm) can be expected in fresh liver material, possibly as a result of diet As the liver becomes more stale tyramine appears, (λ max 275 nm) followed by thymine (λ max 265 nm) which may appear as a metabolite of mould growth When more advanced deterioration sets in, tryptamine (λ max 272, 278, 287) followed by the phenylethylamines (λ max 252, 257 263) start to accumulate in the tissues

The burial in the ground for 14 days of a sample of fresh human liver, which was virtually free of endogenous bases and contained no basic drugs, resulted in the accumulation in the liver of the same putrefactive bases that had previously been encountered The typical UV curves of nicotinamide, thymine and 1- and 2 phenylethylamine were obtained on analysis of the material after internment

This suggests that the pattern for putrefaction is pre-determined by the bacterial content of the liver sample, and is independent of the putrefactive environment

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REFERENCES

Bastos, M L., G E Kananen, R M Young J R Monforte & I Sunshine Detection of basic organic drugs and their metabolites in urine *Clin Chem* 1970, **16**, 931–940

Baker, W, J B Harborne & W D Ollis Characterisation of primary aliphatic amines by reaction with *o* acetyl acetophenol and by paper chromatography *J Chem Soc* 1952, 3215–3217

Clarke, E G C The isolation and identification of alkaloids In *Methods of forensic science* Ed F Lundquist John Wiley and Sons, New York and London 1962, 1, 1–241

Clarke E G C & A E Hawkins A note on the factors affecting Rf values on citrate buffered paper chromatograms *J Pharm Pharmacol* 1963, **15** 390–393

Creveling, C R, K Kondo & J W Daly Use of dansyl derivatives and mass spectrometry for identification of biogenic amines *Clin Chem* 1968, **14** 302–309

Curry, A S The analysis of basic nitrogenous compounds of toxicological importance In' *Methods of biochemical analysis* Ed D Glick John Wiley and Sons Interscience, 1959, 7 39–76

Curry, A S & H Powell The paper chromatographic examination of the alkaloid extract in toxicology *Nature (Lond)* 1954 **173** 1143–1144

Curry, A S J F Read C Brown & R W Jenkins Micro infra red spectroscopy of gas chromatographic fractions *J Chromat* 1968, **38**, 200–208

Curry, A S, R H Fox & P D Evans The identification of alkaloids and synthetic basic drugs *Home Office Central Research Establishment* Report No 36 1970

Dubost, P & S Pascal Determination of largactil in biological fluids Passage through the animal body *Ann Pharm Franc* 1953, **11** 615–619

Dubost, P & S Pascal Determination of chlorpromazine in biological liquids *Ann Pharm Franc* 1955 **13** 56–57

Kæmpe B Interfering compounds and artifacts in the identification of drugs in autopsy material In *Progress in chemical toxicology* Ed A Stolman Academic Press New York and London 1969 4 1–57

McIsaac W M B T Ho, V Estevez & D Powers Chromatography of β carbolines *J Chromat* 1967 **31** 446–454

Nickolls L C Toxicology (chapter 16) In *The scientific investigation of crime* Butterworth London 1956 348 387

Ross J H 2 6 dichloroquinone 4-chloroimide as a reagent for amines and aromatic hydrocarbons on thin layer chromatograms *Anal Chem* 1968, **40**, 2138–2143

Smith T A The quantitative estimation of putrescine by gas chromatography *Anal Biochem* 1970 **33** 10–15

Stevens H M A rapid general screening method for drugs in *post mortem* viscera F Soc 1967 7 No 4 184 192

Truhaut, R & G Le Moan Sur l ınterference des ptomaınes dans l ıdentıfıcatıon des alcaloıdes en toxıcologıe medıco legale *Ann Méd Lég Crımınol* 1958, 38, No 6 394–420

Valov, P Rapıd qualıtatıve and quantıtatıve determınatıon of barbıturates from *post mortem* specımens *Ind Eng Chem (Anal Ed)* 1946 18, 456

Warren, K S & C D Scott Interferıng ultra vıolet absorbıng compounds as sources of error ın common clınıcal chemıstry tests *Clın Chem* 1969, 15, (12) 1147–1156